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EVALUATION OF 5-HALO-6-ALKOXY (OR AZIDO)-5,6-DIHYDRO ANALOGS AS PRODRUGS OF NUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITORS

by

Lili Wang

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

IN

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Dedication to:

My parents, Zhenyie Wang and Nanxiang Zhang

My husband, Hu Liu

My children, Rebecca and Bill

For their love, support and patience

Abstract

AZT, ddI, ddC and d4T are drugs used clinically to combat the HIV virus, the causative agent of AIDS. They act primarily through inhibition of viral reverse transcriptase (RT). However, these drugs are far from being a complete success, because of their dose-related toxicities (such as bone marrow suppression and neuropathy), limited brain uptake, short half-life time in blood and the occurrence of resistance.

5-Halo-6-methoxy (or azido)-5,6-dihydro analogs of AZT, FLT and d4T were synthesized either as putative new anti-HIV agents or as novel prodrugs of AZT, FLT and d4T with enhanced brain uptake. The major focus became the evaluation of these 5,6-dihydro analogs as potential prodrugs.

The 5,6-dihydro analogs are equal or less potent anti-HIV agents than their corresponding parent compounds *in vitro*. Substituents at C^5 and C^6 are factors affecting the activity of the 5,6-dihydro analogs. The 5,6-dihydro analogs are found to be generally more lipophilic than their parent drugs. All of the 5,6-dihydro analogs of AZT and d4T tested to date are resistant to phosphorolysis *in vitro* by *E. coli* thymidine phosphorylase, suggesting that they might be stable *in vivo*. The 5,6-dihydro analogs, in most cases, have increased affinity for the mouse erythrocyte NBMPR-sensitive nucleoside transporter, which may lead to a further increase in their therapeutic efficacy as AZT prodrugs. *In vitro* conversion of the respective 5,6-dihydro analogs to AZT, FLT and d4T upon incubation with glutathione, mouse whole blood or a mouse soluble liver enzyme fraction was found to be dependent upon the nature of the halogen at the C^5 position. 5-Iodo and 5-bromo-6-methoxy-5,6-dihydro analogs convert to their corresponding parent compounds to different degrees in the order of I > Br, whereas 5-chloro-6-methoxy-5,6-dihydro analogs were stable. An *in vivo* investigation of 5-halo-6-methoxy-5,6-dihydro analogs of AZT in mice after i.v. injection demonstrated that the 5-bromo (or iodo)-6-methoxy-5,6-dihydro analogs of AZT (3.6 μmol) provided AZT blood concentrations

similar to those measured with equimolar doses of AZT. On the other hand, the regeneration of AZT from its 5-chloro-6-methoxy-5,6-dihydro analog (6.0 μmol) is about 10 % of that for the bromo or iodo analogs at 15 min after administration, and AZT was not detectable after 90 min. The biodistributions of ^{14}C labeled AZT and its 5-bromo-6-alkoxy (methoxy or ethoxy)-5,6-dihydro analogs, examined in mice after i.v. injection, indicate that these 5,6-dihydro analogs represent a valuable approach for delivery of nucleoside RT inhibitors to the brain. Radioactivity recovered from brain after the [2-^{14}C]-5-bromo-6-methoxy (or ethoxy)-5,6-dihydro analogs was approximately three times that after [2-^{14}C]-AZT was administered. There is no specific subcellular component binding or regional preference associated in brain after [2-^{14}C]-labeled AZT or its 5-bromo-6-methoxy (or ethoxy)-5,6-dihydro analogs are administered. It is concluded that these 5,6-dihydro analogs are potential prodrugs for the clinical treatment of AIDS, providing elevated blood levels and extended plasma clearance properties at equimolar doses, with enhanced plasma/bone concentration ratios. The latter characteristic may provide greater efficacy with reduced toxicity, thereby greatly enhancing their clinical usefulness.

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List of Abbreviations

ADRT	4'-Azido-2'-deoxyribothymidine
AIDS	Acquired immunodeficiency syndrome
AMT	3'-Amino-3'-deoxythymidine
ARC	AIDS-related complex
AUC	Area under the curve
AZdU	3'-Azido-2',3'-dideoxyuridine
AZT	3'-Azido-3'-deoxythymidine
AZTBrOEt	5-Bromo-6-ethoxy-5,6-dihydro-3'-azido-3'-deoxythymidine
AZTBrOMe	5-Bromo-6-methoxy-5,6-dihydro-3'-azido-3'-deoxythymidine
AZTClOMe	5-Chloro-6-methoxy-5,6-dihydro-3'-azido-3'-deoxythymidine
AZT-DP	3'-Azido-3'-deoxythymidine-5'-diphosphate
AZTIOMe	5-Iodo-6-methoxy-5,6-dihydro-3'-azido-3'-deoxythymidine
AZT-MP	3'-Azido-3'-deoxythymidine-5'-monophosphate
AZT-TP	3'-Azido-3'-deoxythymidine-5'-triphosphate
BBB	Blood-brain barrier
BSA	Bovine serum albumin
CA	Capsid
Ci	Curie (s)
μCi	Microcurie
CDS	Chemical delivery system
cib	Concentrative NBMPR-insensitive broadly selective
cif	Concentrative NBMPR-insensitive purine-selective
cit	Concentrative NBMPR-insensitive pyrimidine-selective
cm	Centimeter

CNS	Central nervous system
CPM	Counts per minute
CSF	Cerebrospinal fluid
dATP	Deoxyadenosine-5'-triphosphate
dCTP	Deoxycytidine-5'-triphosphate
ddC	2',3'-Dideoxycytidine
ddG	2',3'-Dideoxyguanosine
ddI	2',3'-Dideoxyinosine
ddN	2',3'-Dideoxynucleosides
ddU	2',3'-Dideoxyuridine
dGTP	Deoxyguanosine-5'-triphosphate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DP	1,4-Dihydro-1-methyl-3-[(pyridylcarbonyl)oxy]
3dT	3'-Deoxythymidine
3dTDP-DMG	3'-Deoxythymidine-5'-diphosphate-3-*sn*-1,2-dimyristoylglycerol
dTDP	Deoxythymidine-5'-diphosphate
dTMP	Deoxythymidine-5'-monophosphate
dTTP	Deoxythymidine-5'-triphosphate
D4T	2',3'-Didehydro-3'-dideoxythymidine
DU	2'-Deoxyuridine
EDU	5-Ethyl-2'-deoxyuridine
EL	Ether lipid analogs
ei	Equilibrative NBMPR-insensitive
es	Equilibrative NBMPR-sensitive
2'-F-ara-ddI	2'-Fluoro-2',3'-dideoxy-2'-arabinofuranosylinosine
2'-F-ara-ddP	2'-Fluoro-2',3'-dideoxy-2'-arabinofuranosylpurine

FBS	Fetal bovine serum
3'-F-ddC	3'-Fluoro-2',3'-dideoxycytidine
5-F-ddC	5-Fluoro-2',3'-dideoxycytidine
FIAU	1-(2'-Fluoro-2'-deoxy-β-D-arabinofuranosyl)-5-iodouracil
FLT	3'-Fluoro-3'-deoxythymidine
FLTBrOMe	5-Bromo-6-methoxy-5,6-dihydro-3'-fluoro-3'-deoxythymidine
5-FU	5-Fluorouracil
FUDR	5-Fluorodeoxyuridine
g	Gram (s)
g	Gravity
GAMT	5'-O-glucuronide-3'-amino-3'-deoxythymidine
GAZT	5'-O-glucuronide-3'-azido-3'-deoxythymidine
GSH	Glutathione
h	Hour (s)
HBS	HEPES buffered saline
HIV	Human immunodeficiency virus
HPLC	High pressure (performance) liquid chromatography
HTLV	Human T-lymphotropic virus
ID_{95}	95% Inhibition dose
i.v.	Intravenous
K_i	Inhibition constant
K_m	Michaelis constant
L	Liter (s)
LAV	Lymphadenopathy-associated virus
M	Molar
MA	Mature matrix
mg	Milligram (s)

min	Minute (s)
mL	Milliliter (s)
mM	Millimolar (s)
mRNA	Messenger RNA
μM	Micromolar (s)
N	Normal
NC	Nucleocapsid
nm	Nanometer (s)
NBMPR	Nitrobenzylthioinosine, 6-[(4-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine
NT	Nucleoside transport
P	Partition coefficient
PBL	Peripheral blood lymphocytes
RNA	Ribonucleic acid
RT	Reverse transcriptase
s	Second (s)
STLV	Simian T-cell leukemia virus
3-TC	3'-Thia-2',3'-dideoxycytidine
Thd	Thymidine
TIBO	Tetrahydro-imidazo[4,5,1-jk][1,4]-benzodiazepin-2(1H)one
TK	Thymidine kinase
$t_{1/2}$	Half-life time
μ	Micro
UV	Ultraviolet spectra
v	Velocity
V_{max}	Maximum velocity
vs	Versus

1. Introduction

1.1. AIDS and HIV.

Acquired immunodeficiency syndrome (AIDS) was first recognized in 1981.[1-3] It is characterized by weight loss, fever, life-threatening opportunistic infections, severe depressed cell-mediated immunity as evidenced by skin anergy, lymphopenia, development of Kaposi's sarcoma and a high mortality rate. Immunologically, there is profound depression of the immune system due to the destruction of a critical component of the immune system, a subset of T cells with important regulatory functions which express the CD4 phenotypic marker (the T4 cell).[4-6] The T4 lymphocyte is known to be the critical cell involved directly or indirectly in the induction of most immunologic functions as described in Figure 1.1.1.[4,7] Although there is not total agreement, it is generally accepted that human immunodeficiency virus (HIV) leads to the depletion of these critical cells. A common and important cause of morbidity in patients with advanced stages of the infection is AIDS dementia complex, a complicating neurological syndrome characterized by abnormalities in cognition, motor performance, and behavior.[8-13] In addition, infection frequently causes a debilitating condition known as the AIDS-related complex (ARC).

Over the years, the number of AIDS cases has escalated extraordinarily.[14,15] The World Health Organization estimated that as of mid-1993 roughly 13 million people have been infected with HIV and that by the year 2000 that number may triple.[16]

Epidemiological studies have shown horizontal transmission by sexual routes and intravenous drug administration, as well as by blood transfusion.[15,17-19] It has also been shown that infants born of infected parents are at high risk.[20-22]

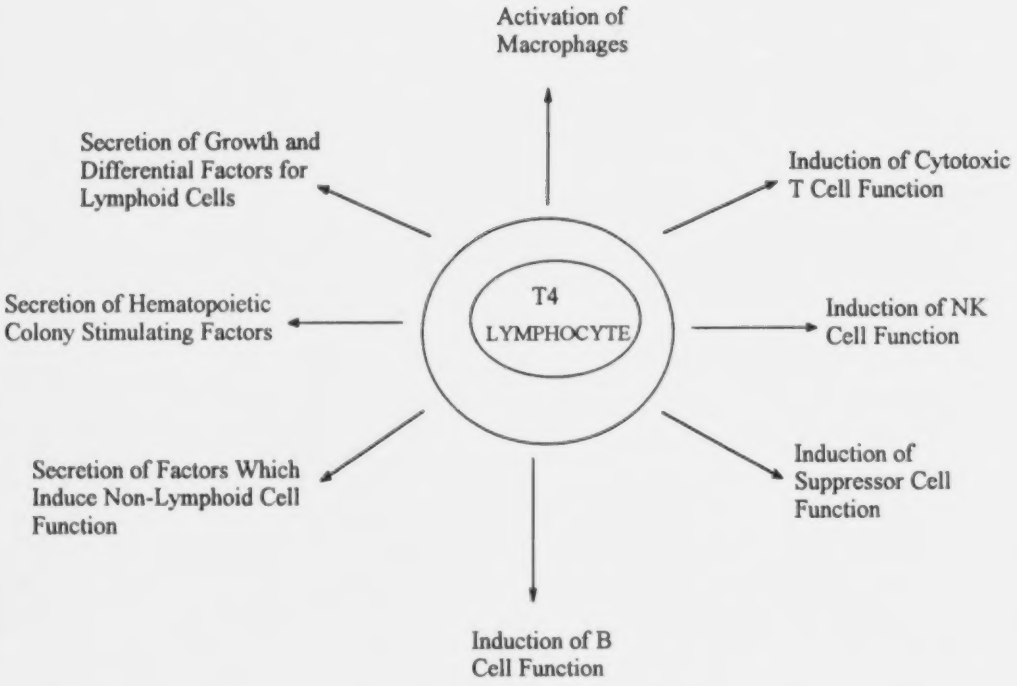


Figure 1.1.1. Critical role of the T4 lymphocyte in the human immune response.[4,7]

Even before the causative agent was identified, the increasing incidence of the disease, the types of patients affected and other epidemiological data suggested the existence of an infectious agent. In 1983, Barré-Sinoussi and colleagues[23] reported the isolation and description of a novel retrovirus, which belongs to the human T-lymphotropic virus (HTLV) family, from a lymph node of a homosexual patient with multiple lymphadenophies, and named it the lymphadenopathy-associated virus (LAV) which was later called HTLV-III.[24] Due to the absence of specific immunological reagents at that time, the link between this virus and AIDS was not recognized.[25] With the availability of large quantities of HTLV-III and the development of specific immunological reagents, this virus, HTLV-III, was finally identified as the primary cause of AIDS.[26,27] HTLVs belong to a single virus group possessing tropism for a subset of helper T lymphocytes. Because of their unique target cell specificity, these viruses, termed HTLVs, destroy the immune system of the infected individual. Depending on the particular virus subgroup, this may lead to abnormal proliferation of functionally impaired helper T lymphocytes (leukemia) or to depletion of the same target cell population (immunosuppression). HTLV-III is the third known virus in the HTLV family and it shares many features with other members of the nontransforming and cytopathic lentivirus family of retroviruses. Of particular note are its morphological, biological, and molecular similarities to the visna virus of sheep,[28] equine infectious anemia virus,[29] and the feline immunodeficiency virus.[30] HTLV-III, the etiologic agent of AIDS, is now called human immunodeficiency virus (HIV)[31,32] which more recently is referred to as HIV-1 to differentiate it from HIV-2 or LAV-2. HIV-2 shares serologic reactivity and polynucleotide sequence homology with simian T-cell leukemia virus (STLV-III) and has been isolated from West African patients with a clinical syndrome indistinguishable from HIV-1 induced AIDS.[33] It has been reported that the envelope glycoproteins of HIV-2 are distinct from the envelope glycoproteins of HIV-1.[33]

HIV virus is considered to be one of the most complicated viruses. In addition to the *gag, pol,* and *env* genes which are the only genes for those replication-competent oncogenic retroviruses, five gene products with regulatory functions (*tat, tev/tnv, rev, nef, vpr*) and two with accessory functions (*vpu, vif*) have been identified either in the virion or in infected cells.[34,35] Knowledge about the interplay of all of these proteins is generating fundamental insight into the underlying basis for virus-mediated pathogenesis in the progress of AIDS.[36] *Gag* codes for the production of mature matrix (MA), capsid (CA) and nucleocapsid (NC), which are associated with formation of virus membrane, viral core shell and encapsidation of genomic viral RNA, respectively. *Pol* gene products (protease, reverse transcriptase and integrase) are responsible for the important enzymatic functions required for HIV-1 DNA synthesis, integration, and processing precursors. *Env* gene product glycoprotein gp120 plays a role in binding of the virus to cell receptor, and gp41 is involved in transmembrane envelope fusion or formation of syncytia. Of the seven additional genes, only two (*tat* and *rev*) have a clearly defined function and are essential to completion of the viral life cycle. *Tat* (transcriptional transactivator) regulates transcription of the proviral DNA to RNA. *Rev* (posttranscriptional transactivator) regulates the conversion from viral precursor mRNA to mature mRNA.

A significant breakthrough was the recognition that the CD4 molecule is an essential component of the receptor on T4 lymphocytes for HIV infection,[37,38] which explains the high tropism of the virus for the T4 cells and the depletion of $CD4^+$ T helper lymphocytes in AIDS.[39] It is now known that other cell types infected via CD4 include macrophages,[40] which explains the infection of tissues like lung and brain.[41] The infection in macrophages may be important because the macrophages can sustain HIV production *in vitro* for a considerable time without being killed, and therefore can serve as a reservoir of HIV. The infected macrophages can subsequently infect other T-cells. Moreover, cytokine secretion by the infection of macrophages can lead to secondary

infection. However, CD4 is not the only receptor for HIV infection. Several $CD4^-$ cell types can be infected by HIV *in vitro*, including epithelial and endothelial cells as well as astroglial, oligodendroglial, and neuronal cells from the central nervous system (CNS) in which galactocerebroside acts as an HIV receptor, although infection of these cell types *in vivo* remains unclear.[43]

The replication of HIV in T lymphocytes is illustrated in Figure 1.1.2.[42,44] After the HIV virion binds to a CD4 molecule on the cell surface, it is internalized and uncoated. The uncoated genomic RNA is released into the cytoplasma and is transcribed into DNA by reverse transcriptase. The genetic information exists in a double-strand DNA form (provirus).[44] The proviral DNA is then integrated into the host chromosomal DNA. After integration of the provirus, the infection may assume a latent phase with restriction of the cycle until the infected cell is activated. Once cell activation occurs, the proviral DNA transcribes viral genomic RNA and messenger RNA (mRNA). Protein synthesis, processing and virus assembly occur, followed by release of a new mature HIV virion.

The virus itself may kill the cells by lysis (causing infected cells to implode or burst) or syncytia formation (fusion of infected cell with non-infected cell with production of multinucleated cells). However, many researchers believe that direct mechanisms are inadequate to explain all of AIDS' devastation. Alternatively, many indirect mechanisms have been proposed to account for the damage caused by HIV infection.

1. Programmed cell death (apoptosis) caused by the infection.[45]
2. Immune responses to infected cells leads to their destruction.[46]
3. Function of the infected cells is changed.[46]
4. Massive increase in permeability of the cell membrane occurs when large amounts of virus are produced and bud off the cell surface.[7]

5. HIV may induce terminal differentiation of the infected T4 cell, leading to a shortened cell life-span.[47]

6. Overproduction of suppressive factors or toxic cytokines.

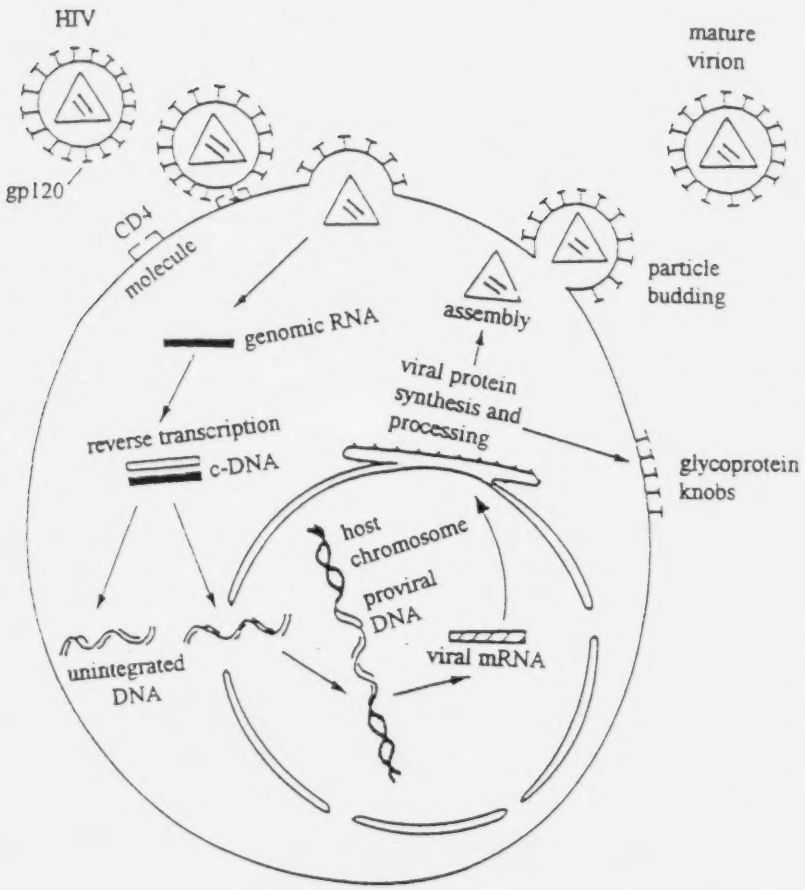


Figure 1.1.2. Life cycle of HIV (adapted from Davita *et al.*[42])

1.2. Molecular targets for AIDS therapy.

Antiretroviral therapy for the treatment of AIDS is based on the assumption that continued retroviral replication is involved in both the pathogenesis and progression of the disease. Whether HIV depresses the immune system by direct or indirect mechanisms, the less virus that is present in an infected person's system, the less damage will be inflicted by the virus. Identification of the molecular events of virus replication has enabled investigators to identify the therapeutic targets for the development of anti-HIV agents.[36,49-53]

The summary presented in Table 1.2.1. indicates that the first step in HIV replication is the attachment of HIV to a $CD4^+$ receptor. Soluble $CD4^+$ molecules bind to virus particles and prevent them from attaching to $CD4^+$ cells; this mechanism represents a possibility for intervention at an early step. In late 1987 and early 1988, several groups reported that recombinant truncated soluble CD4 could inhibit HIV binding and replication in $CD4^+$ target cells *in vitro*.[54-58] A potential advantage of this approach is that it does not cause bone marrow toxicity, which is the main concern for current zidovudine (3'-azido-3'-deoxythymidine, AZT) therapy. However, the recombinant CD4 has a relatively short serum half-life and this poses a drawback.[59] As a result, clinical trials showed either no indication of antiviral activity or only slight activity.[59,60] To address the problem of the short half-life, researchers are exploring altered forms of the soluble CD4. Low molecular mass dextran sulfate (7,000 to 8,000 daltons) has also been shown to block the binding of HIV virions to $CD4^+$ target cells, to inhibit virally-induced syncytium formation, and to exert a potent inhibitory effect against HIV *in vitro*.[61-65] However, clinical trials of orally administered dextran sulfate suggested that this compound has little proven clinical effect.[66] Subsequent research revealed that this compound is poorly absorbed and sufficient inhibitory concentrations are not achieved in

Table 1.2.1. Steps in the HIV replicative cycle that may be targets for therapy.[48]

Step	**Possible Intervention**
Binding to target cell	CD4 analogs Antibodies to HIV or receptor Nonspecific inhibitors
Fusion to target cell	Anti-gp-41 antibodies or drugs that block fusion
Entry and uncoating of RNA	Drugs to block this step of HIV replication
RNA to DNA transcription by reverse transcriptase	Many active drugs (e.g. AZT, and other dideoxynucleosides, phosphonoformate, TIBO compounds) act at this step
Degradation of RNA by RNase	Specific inhibitors of HIV RNase may be found
Migration to nucleus and integration into host DNA	Agents that inhibit these steps may be found
Transcription to translation	Inhibitors of *Tat* and *Rev* activity Anti-sense constructs (e.g. against *tat* and *rev*) TAR decoys (which may bind *tat*) Ribozymes may destroy HIV mRNA
Ribosomal frameshifting	Inhibitors of frameshifting may be discovered
Cleavage of *Gag* and *Pol* polyproteins	Inhibitors of protease
Protein modification	Glycosylation inhibitors (e.g. castanospermine) Myristylation inhibitors may be found
Viral packing	Antisense constructs against the packaging sequence
Viral budding	IFNs may work at other steps as well) Antibodies to viral release antigens Agents that selectively kill cells expressing HIV antigens (eg, CD4-toxin fusion proteins)

plasma.[67,68] In addition to dextran sulfate, antibodies to the CD4 molecule or to gp120 are under consideration as anti-HIV agents.[69,70]

Once HIV is inside the cell, therapeutic strategies to block uncoating would be worth pursuing, but this approach has received little attention. The aromatic polycyclic dione compounds hypericin and pseudohypericin were shown to be active against HIV-1 *in vitro*.[71] These plant-derived materials may act in part at this step, but clinical results are not available yet.

However, the next step in viral replication which involves transcription of viral RNA to DNA, has been a major target for the development of anti-HIV agents. The production of a single complementary strand (first strand) DNA copy of the viral RNA genome, followed by the production of a second (positive strand) DNA copy, is catalyzed by reverse transcriptase (RT) which requires four substrates (dATP, dGTP, dCTP, and dTTP), a primer, a template, and a number of cofactors. RT activity is essential for HIV replication and is considered a unique viral enzyme. Moreover, there is evidence that if a DNA copy of uncoated HIV RNA genome is not made promptly, the RNA is susceptible to degradation by cellular enzymes. The virus also degrades genomic RNA (using RNase H) as part of the process of reverse transcription. Therefore, inhibition of RT activity can potentially prevent infection of a cell by HIV. Nucleoside analogs were a logical first choice among which to search for anti-HIV agents. In fact, much of the early work devising therapy against HIV has focused on inhibiting RT.[72-75] Potent anti-HIV agents were identified from the screening of a number of nucleoside analogs previously made for anti-cancer therapy. Many additional potent and selective HIV RT inhibitors including nucleoside analogs have subsequently been made.[51,76,77] They are classified into two groups. Members of the first group, comprised of 2',3'-dideoxynucleosides (ddNs), carbocyclic and acyclic nucleosides, are considered to act as competitive inhibitors. They need to be intracellularly phosphorylated to their triphosphate forms before they interact

competitively with RT. The second group of HIV inhibitors includes the tetrahydro-imidazo[4,5,1-jk][1,4]-benzodiazepin-2(1H)one (TIBO) derivatives and TIBO-like compounds. The compounds in this group do not require any intracellular activation and they noncompetitively inhibit RT activity due to their specific interaction with HIV-1 RT.[51]

Many of the compounds targeted at RT including both nucleoside and non-nucleoside analogs, such as 3'-thia-2',3'-dideoxycytidine (3-TC) and R-82913, have entered clinical trials, in addition to the four agents (AZT; 2',3'-dideoxyinosine, ddI; 2',3'-dideoxycytidine, ddC; and 2',3'-didehydro-3'-deoxythymidine, d4T) approved for clinical use.

Subsequent integration of viral DNA into cellular DNA is mediated by HIV endonuclease (integrase). This enzyme is a possible target for therapy, and specific inhibitors of *pol*-encoded integrase may be found.

The integrated viral DNA is then transcribed by cellular polymerases and translated by the cellular machinery to viral proteins. HIV protease is essential for proper virion assembly and maturation. Inactivation of HIV protease by either mutation or chemical inhibition leads to the production of immature, non-infectious viral particles. Thus, HIV protease is considered to be a promising target for the rational design of drugs against AIDS, and HIV protease inhibitors represent another class of therapeutic agents that complements existing approaches to antiviral therapy that target HIV RT.[78,79] Protein crystallography has been particularly useful in helping to elucidate precise interactions between these HIV protease inhibitors and their target enzymes, and has been involved in protease inhibitors research.[80] In this category, very potent and selective *in vitro* inhibitors have been synthesized and their interactions with the protease have been studied by X-ray crystallography.[80] The first inhibitors of protease, the peptide-based substrate analogs, were reported to be active *in vitro*. However, the first inhibitor which entered a

clinical trial, Ro 31-8959, showed fairly low oral solubility and low bioavailability,[81] because of its peptide nature. Development of resistance to this class of protease inhibitors has also been recognized.[82] Recent studies gave rise to second generation inhibitors, such as A-77003 and A-80987, with improved oral bioavailability and serum half-life in animals; A-80987 is currently in a phase I trial.[83] More recently, nonpeptide cyclic ureas have been designed as more potent and bioavailable protease inhibitors.[84] Animal studies have confirmed the design and further studies, including a phase I trial, on DMP 323 are under investigation. Processing of HIV proteins also involves extensive glycosylation and myristylation, which are considered potential targets. Several HIV-coded regulatory proteins participate in HIV replication and the transactivating proteins *tat* and *rev* especially are being utilized as targets in the search for new inhibitors.

Assembly and release of virus particles are also virus-specific processes which are targets for inhibitors, but again little has been reported on the development of drugs to interfere with these processes.

1.3. 3'-Azido-3'-deoxythymidine (AZT) and related compounds.

Although virtually every step in the replication cycle of HIV could serve as a target for a therapeutic intervention, HIV RT inhibitors, especially nucleoside analogs, have been the most extensively studied. All four AIDS antiviral drugs marketed today are nucleoside analogs, namely AZT (also known as Zidovudine, Retrovir), ddI (also known as Didanosine, Videx), ddC (also known as Zalcitabine, HVID) and d4T (also known as Stavudine, Zerit) (structures shown in Figure 1.3.1). In 1984, during the screening of previously synthesized nucleoside analogs, AZT, which was first synthesized in 1964 by Horwitz,[85] was identified as an active agent against HIV in cell culture.[73] In 1985,

clinical studies began. In phase I studies (safety and feasibility), AZT was found to be well tolerated over a six-week period.[74] Some subjects showed clinical and immunological improvement characterized by an increase in the number of T helper cells, development of delayed hypersensitivity reactions and weight gain. Then a multicentred double-blind, placebo-controlled trial was conducted in a total of 282 patients.[86] The trial was prematurely terminated because a significant difference in death rate was observed between AZT and placebo recipients. Decreased mortality and frequency of opportunistic infections during a period up to 6 months were achieved in the AZT-treated group. In other studies, neurological dysfunction was shown to be partly reversible upon AZT treatment.[87-89] In 1987, AZT was licensed.[90] Subsequently, AZT administered earlier in the progression of the disease state was shown to delay the onset of AIDS-associated opportunistic infections.[91,92]

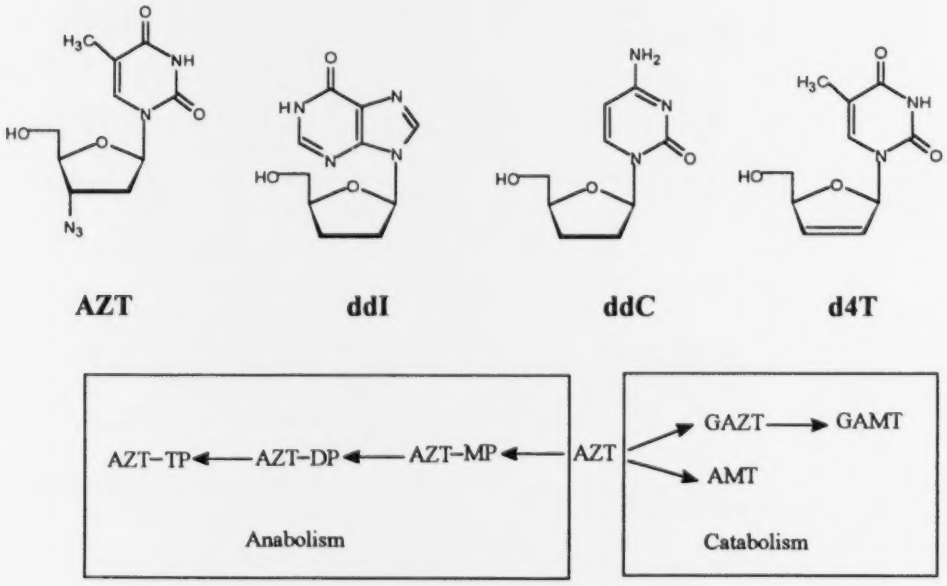


Figure 1.3.1. The chemical structures of AZT, ddI, ddC and d4T, and the metabolic pathways of AZT.

The cellular metabolism of AZT and the mechanism of its function are well documented. AZT is subject to both anabolism and catabolism as shown in Figure 1.3.1.

AZT is a good substrate for cellular thymidine kinase (TK) and it is phosphorylated as efficiently as thymidine. Mono-, di- and tri- phosphates (AZT-MP, AZT-DP and AZT-TP) were observed by HPLC analysis, with predominance of the monophosphate (AZT-MP). It appears that the phosphorylation from mono- to di-phosphate is the rate-limiting process due to the inhibition of dTMP kinase activity by accumulation of AZT-MP as shown in Figure 1.3.2.[93,94] The rate of phosphorylation is substantially lower in resting monocytes/macrophages than that in T-cells as demonstrated by Perno[95] and Richman,[96] where the nucleoside kinase activity is known to be low. Following intracellular conversion to its triphosphate (AZT-TP), two antiretroviral mechanisms of action have been proposed: (1) competitive inhibition of normal substrate (dATP, dGTP, dCTP, and dTTP) binding to the retroviral RT and (2) incorporation of the AZT-TP into retroviral DNA, resulting in DNA chain termination[73,75,93,97-99] because of the absence of the -OH at the 3'-position (see Figure 1.3.2.). It is not known, however, which of these mechanisms is more important or

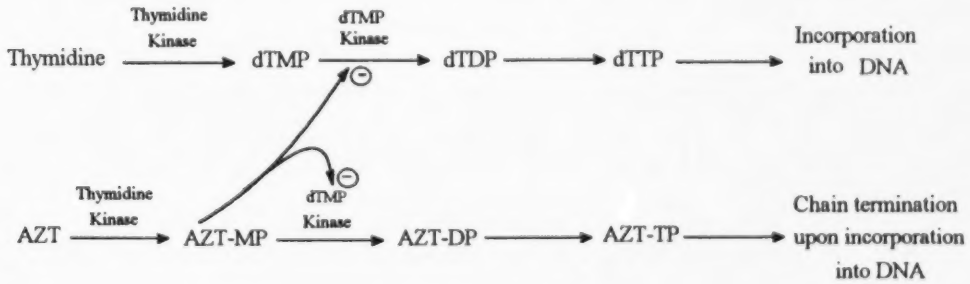


Figure 1.3.2. Intracellular anabolism of thymidine and AZT.

whether either of these actions alone is sufficient to permanently block retroviral reverse transcription. In addition to its interaction with viral RT, AZT also interacts with cellular

polymerase. In this regard, it has been reported that the cellular DNA polymerase alpha is 100 times less susceptible to inhibition by AZT-TP than is HTLV-III RT.[93] This suggests that AZT is nonselectively phosphorylated, but that AZT-TP binds to HIV RT more efficiently and selectively than to cellular polymerase. The dose that causes 95% viral inhibition (ID_{95}) *in vitro* is below 1 μmol/L for most clinical isolates.[100]

The bioavailability of AZT after oral administration is about 63%.[101] Metabolism studies indicated that three metabolites, 5'-O-glucuronide-3'-azido-3'-deoxythymidine (GAZT), 3'-amino-3'-deoxythymidine (AMT), and 5'-O-glucuronide-3'-amino-3'-deoxythymidine (GAMT) are formed (structures shown in Figure 1.3.3.), with GAZT as the predominant metabolite.[102-105] GAZT has also been identified in other species such as rats, mice and dogs, but accounts for less than 5% of the administered AZT dose.[106]

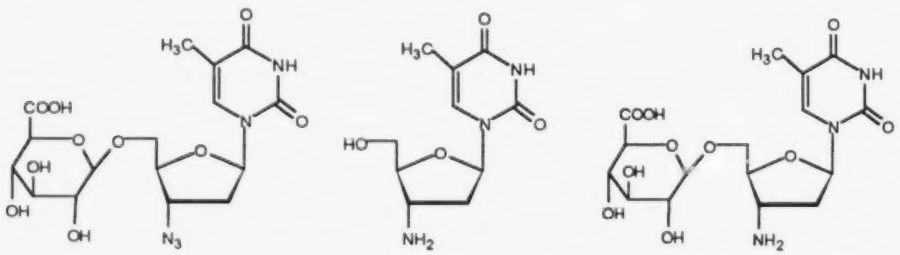


Figure 1.3.3. The chemical structures of AZT metabolites.

Despite the growth in use of AZT since 1987, its optimal dose has yet to be precisely defined. Originally AZT was used at a dose of 1500 mg/day, a dose that causes bone marrow toxicity in 30 to 45% of patients. Those patients required transfusions or a reduction of dosage. The recommended dose is now 500 to 1000 mg/day in patients with AIDS and ARC, in asymptomatic patients with CD4 < 200/mm^3 and in asymptomatic

patients with CD4 between 200 and 500/mm^3 with a rapid decrease of CD4 cell count or a positive p24 circulating antigen.[107]

AZT is far from being an ideal agent for the treatment of AIDS. AIDS still remains a fatal disease, and many problems have been encountered with AZT usage.

1. Toxicity. In the first clinical trial, side effects, especially serious bone marrow suppression resulting in anemia and neutropenia, were noted.[108] For some patients, either the regular dose had to be reduced or the treatment had to be terminated because of the bone marrow toxicity. In a later study, AMT was identified as a toxic catabolite produced by hepatocytes and liver microsomes; this metabolite was 5- to 7-fold more toxic than AZT.[111] The toxicity is also likely due to an AZT-induced deficiency of dTTP as shown in Figure 1.3.2.[93] Another major complication of continuous usage of AZT is development of myopathy which is characterized by myalgia, muscle weakness and elevated levels of serum creatine kinase.[109,110] This myopathy poses a significant morbidity to patients who receive AZT and limits therapy. This toxic effect is generally improved when AZT is discontinued. Studies have shown that after 35 days of treatment AZT caused selective changes in rat skeletal striated muscle which localized ultrastructurally to mitochondria, and which induced swelling, cristae disruption and mitochondrial myelin figures with whorled lamellae. Associated with the ultrastructural changes were decreased muscle mitochondrial DNA (mtDNA), mtRNA, and mitochondrial polypeptide synthesis *in vitro*, suggesting that AZT-induced inhibition of mtDNA replication mediated by interference with DNA polymerase γ has an effect of depressing the abundance of striated muscle mtDNA, mtRNA and mitochondrial polypeptide synthesis.[112-116] Serious toxicities can be limited by lower doses, but this protocol is not adequate for treating children or for patients with cerebral infection.[118] Increased antigenemia, recurrent symptoms, or decreased CD4+ cell counts have been reported with subtherapeutic doses of AZT.[119] Bone marrow toxicity has been

recognized as a common side effect associated with nucleoside-type RT inhibitors. Non-nucleoside RT inhibitors have been studied because of their high activity and low toxicity. However, these compounds have shown a high rate of resistance development. Patients given the TIBO analogs, nevirapine and L-697661, develop resistance within a few weeks.[120]

2. Fast clearance. Although AZT would not be anticipated to decrease the production of virus from cells already infected with HIV, effective concentrations should protect new cells from becoming infected. However, disease progression occurs during antiretroviral therapy. The failure of existing drugs to stop the progression of AIDS is due in part to their inability to maintain adequate drug levels at the site of replication over extended periods as result of their relatively short half-lives. Pharmacokinetic studies[74,121] suggested that the elimination $t_{1/2}$ in human plasma was only about 1 h and the peak plasma concentration following a 250 mg oral dose was 2.3 μmol/L. The short half-life makes frequent dosing necessary. Although the half-life of intracellular AZT-TP is 3 to 4 h, the correlation between intracellular AZT-TP and plasma AZT concentrations is not clear.[122] Therefore current anti-HIV therapies, once initiated, require frequent administration of drug owing to the relatively short half-life of AZT and usually continue throughout life.

3. Limited brain uptake. Since the first observation that the brain is an important site of HIV replication,[123] there has been a growing recognition that serious neurologic dysfunction, ranging from peripheral neuropathy to profound dementia, may be caused by the virus.[11,116-129] Viruses have been isolated from the cerebrospinal fluid (CSF) and brain tissue.[41,124,130-132] Although it is still not known precisely how HIV enters the nervous system (one possibility is that it is transported there by infected macrophages[133]) or how it induces neurologic damage, it is clear that successful therapeutic strategies must deal with the phenomenon of viral replication within the nervous system. It has been

reported that more than 50% of children with AIDS suffer progressive encephalopathy and about 65% of adult patients develop dementia. AZT can penetrate into the CSF,[74,121] and the concentration of AZT in the CSF has been reported to be dose-related, being approximately 35 and 70% of the plasma concentration at the dose of 2.5 and 5 mg/kg, respectively,[121] which suggests that there is a direct correlation between the decline in CSF/plasma ratios and decreasing dose. However, the transport of AZT into CSF does not mean the penetration of AZT through the blood-brain barrier (BBB). The amount of AZT crossing the BBB is likely not sufficient to provide a therapeutic concentration that suppresses viral replication in the brain.[134] Less than 1% of AZT injected via the carotid artery in rats penetrated into brain.[135]

4. Resistance. Although AZT has been shown to improve the quality of life, or prolong the life of patients with AIDS, the beneficial effect in most cases is transient, symptoms eventually reappear, and the infections become refractory to therapy. A number of groups[100,138-142] have reported that viruses isolated from patients treated with AZT for prolonged periods of time are more resistant to AZT than viruses taken from the same patients prior to the therapy. This resistance phenotype has been found to correlate with *pol* gene mutations which cause amino acid substitutions at five positions in the RT.[143,144] Mutations at one or more positions resulted in significant resistance. Possible strategies for preventing, or delaying the development of resistance include change or combination therapy with two or more drugs. Reports of synergy[145-148] and lack of cross-resistance between RT inhibitors,[146,149-151] plus the reversal of AZT resistance induced by ddI and non-nucleoside RT inhibitors[152] have supported the concept of change or combination therapy with more than one RT inhibitor. An AZT-resistant strain isolated from patients was shown to be susceptible to other nucleoside analogs including ddI and ddC, and non-nucleosides, such as interferons.[146] Cross-resistance was only observed with nucleoside analogs containing a 3'-azido moiety. It has also been noted that

sensitivity to AZT can be recovered by discontinuation of AZT and initiation of ddI therapy.[149] These results suggest that other RT inhibitors, such as ddI, can be used when AZT shows resistance after prolonged treatment. In addition, combination strategies may allow the use of individual agents below their toxic concentrations, provide more complete viral suppression and limit the emergence of drug-resistant mutants.[153]

The discovery of the above problems (toxicity, short half-life, limited brain uptake as well as resistance) associated with AZT has initiated an intensive effort to develop other agents with better efficacy and selectivity. In addition to AZT, ddI, ddC and d4T have been approved for clinical use.

ddI produces a moderate rise in CD4 cell number, is less toxic,[155,160] and is used for individuals who have been on AZT for 4 months or longer or who are hematologically intolerant to or no longer respond to AZT. Major side effects of ddI include acute pancreatitis and peripheral neuropathy,[161] and the side effects observed for AZT including bone marrow toxicity. ddC is used in combination with AZT.[156] Resistance to both ddC and ddI have been reported, however cross-resistance to AZT was not observed in both cases.[149,162] d4T has recently been approved for HIV-infected adults who no longer respond to or cannot tolerate the other three approved anti-HIV drugs. d4T is about 20- to 100- fold less toxic to human granulocyte macrophage progenitors than AZT. Unlike AZT, d4T is a good substrate for thymidine phosphorylase, which effects the cleavage of the glycosidic bond as shown in Figure 1.3.4. However, intracellular thymidine phosphorylase levels vary in tissues and lower level is found in T lymphocytes than in bone marrow cells. The lower toxicity is believed to be due to fast phosphorolysis of d4T in bone marrow. A major side effect with d4T is peripheral neuropathy. The penetration of d4T into the CNS was found to be low in mice.[164,165]

It has been recognized that the continuous usage of these compounds is essential for the suppression of HIV replication in patients with AIDS. However, toxicities

associated with continuous usage of these compounds limited their long term treatment. Bone marrow suppression, which generally occured within 4 weeks, was one of the major toxicities in AIDS patients who received AZT therapy, whereas patients who took continuous doses of ddC developed a painful peripheral neuropathy after 8 to 12 weeks.[166] A similar delayed toxicity to the peripheral nerve system was also reported in patients treated with ddI and d4T.[167] It has been shown that these ddNs caused mitochondrial toxicity characterized by profound inhibitory effects on mtDNA synthesis.[115,116] The mitochondrial defects have been related to peripheral neuropathy and many other diseases.[117]

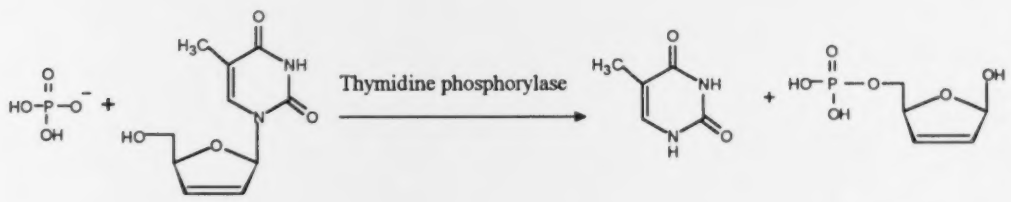


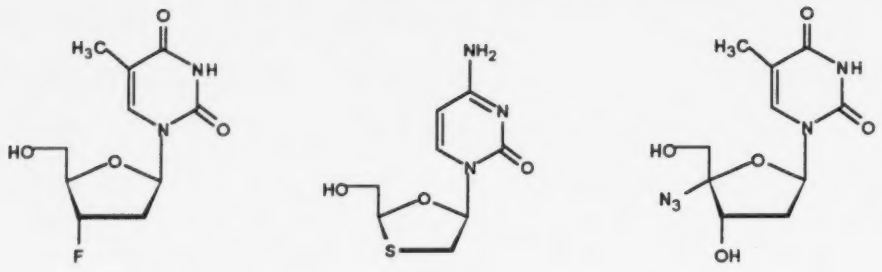
Figure 1.3.4. Phosphorolysis of d4T.

Like AZT, ddI, ddC and d4T all function to inhibit HIV RT.[155,156]

More compounds have been identified and entered into clinical trials. Some of the nucleoside analogs which are in clinical trial are summarized in Table 1.3.1. and their structures are shown in Figure 1.3.5.

Nucleoside RT inhibitors which have gone to clinical trials include FLT, 3-TC and ADRT.

3'-Fluoro-3'-deoxythymidine (FLT) has been reported to be one of the most potent inhibitors of HIV *in vitro*, but it is toxic at a 1-100 μM concentration depending on the cell culture system used.[163] AZT-resistant viruses are not cross-resistant to FLT. Clinical



FLT **3-TC** **ADRT**

Figure 1.3.5. The chemical structures of some of the nucleoside analogs.

Table 1.3.1. Anti-HIV nucleosides in clinical trials.

Drug	Chemical name	Clinical status
AZT	3'-Azido-3'-deoxythymidine	Approved[154]
ddI	2',3'-Dideoxyinosine	Approved[155]
ddC	2',3'-Dideoxycytidine	Approved[156]
d4T	2',3'-didehydro-3'-deoxythymidine	Approved[157]
FLT	3'-Fluoro-3'-deoxythymidine	Terminated after Phase II
3-TC	3'-Thia-2',3'-dideoxycytidine	Phase I/II[53]
ADRT	4'-Azido-2'-deoxythymidine	Preclinical development[159]
5-F-ddC	5-Fluoro-2',3'-dideoxycytidine	Phase I[159]

studies suggested that thrombocytopenia and anemia are the main limiting toxic effects, and a narrow therapeutic window has been observed. The further development of FLT

was recently discontinued because significant antiviral activity was not observed at plasma concentrations that were well tolerated in a phase II study.

4'-Azido-2'-deoxythymidine (ADRT) is an unusual nucleoside RT inhibitor because it retains the OH group at the 3'- position. ADRT is incorporated into DNA and it is a chain terminator despite the presence of a 3'-OH, but only when two ADRT molecules are incorporated adjacent to one another. AZT-resistant viruses are not cross-resistant to ADRT.

3'-Thia-2',3'-dideoxycytidine (3-TC) is an analog of ddC. Its β-L-(-)-isomer is more potent than the β-D-(+)-isomer which undergoes substantial deamination by cytidine/deoxycytidine deaminase.[168] 3-TC is reported to be less toxic than AZT[169] and it is not cross-resistant to AZT. It is significant that 3-TC is also effective against human hepatitis B virus.[170,171]

The recognition that current therapies do not completely suppress disease progression and have associated toxicities has served as a stimulus for expanded research to identify more efficient drugs. The search for more effective anti-HIV therapies includes discovering new drugs with different mechanisms of action,[61,71,79,172] and developing prodrugs to improve those drugs already shown to be efficacious, particularly AZT.

1.4. Nucleoside transport.

The importance of membrane permeation by nucleoside drugs as an initial determinant of their intracellular anabolism and hence their therapeutic activity has been recognized previously.[173-180]

Biological membranes are comprised of lipids and proteins, together with smaller amounts of associated carbohydrates. Different membranes vary greatly in their relative

proportions of lipids and proteins. However, all membranes are composed of a matrix of lipid molecules into which proteins are inserted, and other proteins may be attached to this fundamental structure (shown in Figure 1.4.1.).[181] The lipids are almost always fluid. The head groups pack tightly together but the hydrocarbon tail regions are very flexible. If a molecule is to cross this region of the membrane, it must first enter the lipid region, cross it and exit at the other face of the membrane. To enter the lipid region is to enter an hydrophobic environment. The mechanisms involved in the transmembrane movements include simple diffusion, a channel model, facilitated diffusion, a co-transport system and active transport processes.

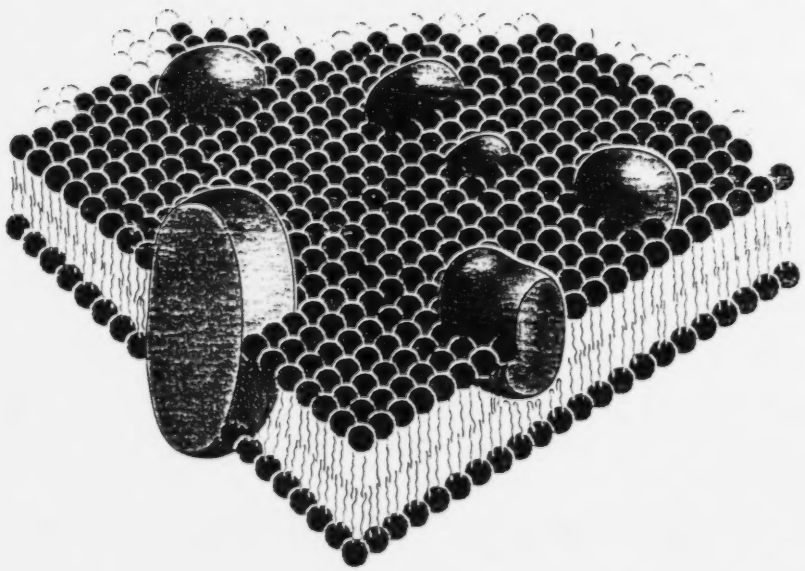


Figure 1.4.1. Fluid mosaic model of a membrane taken from Singer and Nicolson.[181]

Pyrimidine ribonucleosides and deoxyribonucleosides are physiological molecules which are required for RNA and DNA synthesis, respectively. Modifications of the sugar moiety and uracil base of natural nucleosides have provided many compounds which are active antiviral or antitumor agents. These nucleosides are phosphorylated by kinases to nucleotides which are inhibitors of specific enzymes. However, transport into cells is an important determinant of the biological activity of nucleoside drugs, because all enzymes which activate the nucleosides are inside the cell. Thus, it is important to understand the transport mechanisms for these nucleosides. It is well known that nucleoside permeation of mammalian cell membranes is complex and involves several distinct transporters. These transport processes can be classified into two classes deponding on whether they involve equilibrative or concentrative transport of nucleosides across membranes.[182] The equilibrative transport processes exhibit the typical features of the facilitated diffusion and are driven by the concentration gradient of nucleosides being transported. The concentrative transport processes are driven by transmembrane Na^+ gradients and are inwardly directed Na^+/nucleoside cotransporters. Equilibrative nucleoside transporters are widely distributed among mammalian cells and tissues, whereas concentrative nucleoside transporters are limited to specialized cell types including intestine, kidney, spleen lymphocytes, macrophages and choroid plexus.

Two equilibrative transporters have been well documented.[183,184,202,204] They differ in their sensitivity to 6-thiopurines, such as NBMPR (6-[(4-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine)[183] (structure shown in Figure 1.4.2.). The equilibrative transporter, which is sensitive to 6-thiopurines, is termed *es* and is inhibited by nanomolar concentrations of NBMPR.[185] Human erythrocytes[183-188] and leukocytes[189-191] possess this type of nucleoside transporter. The other equilibrative transporter, which is insensitive to 6-thiopurines and not affected by NBMPR at concentrations up to 1 μM, is

called *ei* and has been identified in L1210 murine leukemia cells and other cultured neoplastic cells.[183,184,192]

On the basis of substrate selectivity, at least three Na^+-dependent transporters have been identified in several normal tissues and in one neoplastic cell line.[193-199] One system, designated as *cif*, is selective for purine nucleosides and formycin B is a model substrate. The *cit* concentrative Na^+-dependent nucleoside transporter is selective for pyrimidine nucleosides and thymidine serves as a model substrate. The above two systems exhibit some overlapping substrate specificities. For example, uridine appears to be a substrate for both *cif* and *cit* systems. The third Na^+-dependent nucleoside transporter, termed *cib* which accept both purine (guanosine, inosine and formycin B) and pyrimidine (uridine and thymidine) nucleosides, has been demonstrated in rabbit choroid plexus. All these Na^+-dependent transporters are insensitive to 6-thiopurines.

Physiological nucleosides, and many of their analogs, enter mammalian cells by the equilibrative, facilitated diffusion nucleoside transport (NT) processes and/or by the concentrative, Na^+-dependent NT processes.[200,201] These processes appear to be saturable and subject to competitive inhibition by other nucleosides. Antiviral ddNs [such as AZT, 3'-deoxythymidine (3dT), ddA, ddI, ddC and d4T] have been reported to enter cells either predominantly or partially by nonfacilitated diffusion due to their relatively increased lipophilicity [AZT, P (partition coefficient) = 1.26;[173] ddT, P = 0.20;[177] d4T, P = 0.23;[178] and ddA, P=0.61].[179] Recently, in human erythrocytes and human T-lymphoblastoid CCRF-CEM cells, a nucleobase transporter which is inhibited by adenine, hypoxanthine and acyclovir was found to facilitate the entry of some ddNs such as 2',3'-dideoxyguanosine (ddG) and ddI, into these cells.[202,203]

As the first and still the most important anti-HIV agent, AZT has been extensively studied in regard to crossing cell membranes. Zimmerman *et al.*[173] reported the unusual ability of AZT to cross cell membranes by passive diffusion. First, the initial velocity of

AZT influx was directly proportional to AZT concentration, over the range of 0.5-10 mM. Second, several potent inhibitors (NBMPR, dipyridamole and dilazep) of nucleoside transport were without effect on the initial velocity of AZT influx. Third, several nucleosides (thymidine, uridine and 2-chloroadenosine), which are known to be permeants of the nucleoside transporters, had no effect upon the rate of AZT influx when tested at a 1000-fold molar excess relative to AZT. Finally, the temperature coefficient (Q_{10}) for AZT influx into erythrocytes was only 1.4, compared with a Q_{10} of 2.7 for thymidine. Studies using renal basolateral membrane vesicles from rat have demonstrated involvement of the organic anion transport system in the transport of AZT across the basolateral membrane.[174] AZT has been shown to inhibit the organic anion transporter and to stimulate counter flow of p-aminohippurate (a substrate for the organic anion transporter), suggesting that AZT transport in renal basolateral membrane vesicles is mediated by the renal organic anion transport system. More recently, a complementary DNA encoding *cit*-type Na^+-dependent nucleoside transporter (named cNT1) has been cloned and it was found that AZT is accepted as a permeant by this system.[175,176]

One tissue in which improved passive permeability can lead to significant advantages is drug delivery to the brain through the BBB.[136,137] Entry of drugs into the CNS through the BBB is difficult because, unlike systemic capillaries, the endothelial cells of CNS capillaries have very tight junctions and are devoid of intracellular spaces. The endothelial cells are also relatively devoid of pinocytotic activity. Therefore, drugs which are capable of entry into systemic tissues enter the CNS less readily. Various strategies, to be discussed later, have been employed to design prodrugs to improve the delivery of AZT and selected ddNs to the brain.[205-209]

With respect to passive diffusion, the ability of a molecule to diffuse across the lipid bilayer is related to its partition coefficient. Therefore, many nucleoside prodrugs with increased lipophilicity have been studied in attempts to improve their passage across

the BBB and into the brain. On the other hand, it has been suggested that the levels of AZT and other ddNs in CSF and brain depend on not only the influx but also the efflux of these ddNs, and efflux appears to be the predominant factor in determining the net accumulation of respective drugs into CSF and brain. It has been demonstrated in rats that the overall rate constant for the efflux of AZT from brain was reported to be approximately 75-fold higher and from CSF is 8-fold higher than the respective rate constants for influx.[210] The low levels of AZT in CSF and brain have therefore been attributed to its active transport out of the CNS. It has also been reported that coadministration of probenecid (an inhibitor of organic anion transport) increased AZT AUCCSF by sevenfold in rabbits, suggesting that AZT is transported out of CSF by the organic anion transport system.[211] Microdialysis study in rabbits further confirmed that probenecid decreased the clearance of AZT from CSF and thalamus extracellular fluid (ECF), prolonging the half-lives of AZT disappearance from the brain. Probenecid elevated the AZT AUC in the ventricular CSF 3- to 5-fold and the AUC in the thalamus ECF by 5- to 6-fold, whereas the AUC of plasma increased only about 2-fold, indicating that AZT is actively transported outward across the CSF- and brain ECF-blood barriers, and this transport system is sensitive to probenecid.[212]

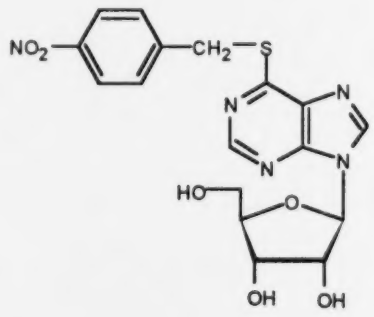


Figure 1.4.2. The chemical structures of NBMPR.

1.5. Prodrugs to nucleoside inhibitors of reverse transcriptase.

A prodrug is a pharmacologically inactive compound which is converted to an active drug by metabolic biotransformation. The prodrug approach is used to solve pharmaceutical problems (such as stability, solubility), pharmacokinetic problems (such as retention time in plasma) or drug delivery problems (such as specific-site targeting), by chemical modification of an active drug (parent drug).[213-215]

An ideal prodrug should fulfill certain requirements, which include ready conversion from the prodrug to active drug *in vivo*, adequate *in vitro* stability, low toxicity and other desired characteristics such as specific delivery and improved physico-chemical properties. The prodrug-to-drug conversion can occur before, during, after absorption, or at a specific site in the body. However, in an ideal case, the prodrug-to-drug conversion should take place as soon as the specific goal of making the prodrug is achieved, since the prodrug is inactive. For example, a prodrug designed to solve the solubility problem for making an intravenous injection solution should convert to the parent drug once it is in the body. If a prodrug is designed to produce a sustained drug action, the rate of conversion should match the desired rate of release from the formulation.

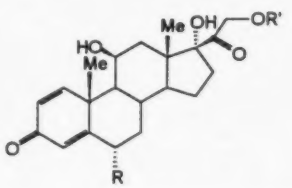
1.5.1. Types of prodrugs.

1.5.1.1. Esters.

The most common prodrugs studied are esters of active drugs. Active drugs containing a hydroxyl or carboxyl group can be readily converted to their ester prodrugs (Drug-OCOR or Drug-COOR). The regeneration of the active drug requires hydrolytic cleavage by endogenous esterases (Drug-OCOR → Drug-OH + RCOOH; or Drug-COOR → Drug-COOH + ROH). The popularity of esters as prodrugs is due to the fact that esterification is a simple and effective way to obtain prodrugs with a wide range of

lipophilicities and conversion rates, and the body is rich in esterases. The distribution of esterases in the body is ubiquitous, and all mammalian tissues can contribute to the hydrolysis of esters. The liver, the gastrointestinal tract, and blood have the greatest hydrolytic capacity.[213-215] However, the specificity of various esterases toward variations in substrate structures is poorly understood.

As an example of ester prodrug approach to improve drug delivery we can look at methylprednisolone which has a poor water solubility. In order to permit aqueous injection or ophthalmic delivery, its prodrug, methylprednisolone sodium succinate, was developed.[215]



$R=CH_3$, $R'=H$; Methylprednisolone

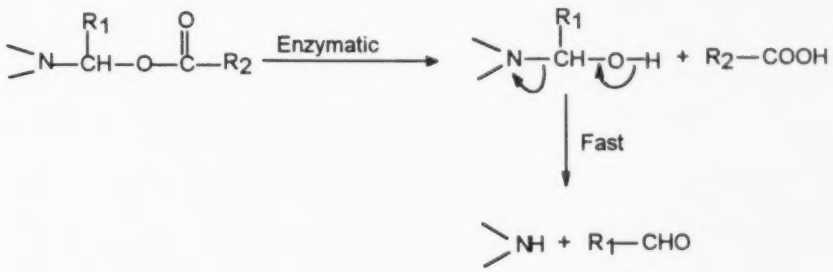
$R=CH_3$, $R'=COCH_2CH_2CO_2Na$; Methylprednisolone sodium succinate

1.5.1.2. Amides.

Drugs containing an amine group can be converted to amides. However, this approach is not commonly used because of the stability of amides toward hydrolytic cleavage. Activated amides, such as N-Mannich bases (Drug-NH-CH_2NHCOAr), phenyl carbamates (Drug-NH-CO_2Ph) or amide derivatives of amino acids, have elevated susceptibility to enzymatic cleavage.

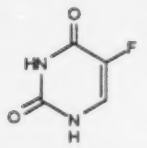
Since the early 1980's, N-acyloxyalkylation has become a common approach to obtain prodrugs of various amides, imides, hydantoins, uracils, tertiary or N-heterocyclic amines.[216-219] The usefulness of this approach relies on the fact that by varying the acyl portion of the derivatives it is possible to control the rate of regeneration of the parent drug and to obtain prodrugs with varying physicochemical properties such as water-solubility or lipophilicity. They are rapidly cleaved *in vivo* by enzyme-mediated

hydrolysis. The regeneration of the parent NH drug takes place via a two-step process shown below:

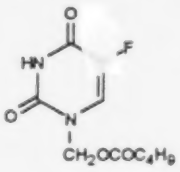


Enzymatic cleavage of the ester results in the formation of an N-hydroxyalkyl derivative which subsequently is assumed to decompose instantaneously into the corresponding aldehyde and the NH drug. Thus the rate of regeneration of the drug is dependent on the rate of the hydrolysis of the ester, which can be controlled by steric and electronic factors. Several papers have described N-acyloxyalkylation as a means of obtaining prodrugs of theophylline,[220] 6-mercaptopurine[221] and 5-fluorouracil (5-FU)[222] to enhance the dermal delivery of these compounds. The N-acyloxyalkylated prodrugs have increased lipophilicity and they are susceptible to enzymatic cleavage in the skin. As an example, 5-FU has proven to be a valuable topical agent. However, it has poor aqueous solubility (2.5 mg/mL) and low lipophilicity (log P = −1.00) for achievement of optimal topical bioavailability. The poor solubility of 5-FU in both water and organic solvents is largely a result of the high crystal lattice energy in the molecule due to intermolecular hydrogen bonding formed between NH and C=O moieties. 1-Butyryloxymethyl-5-fluorouracil (1-butyryloxymethyl-5-FU) and 1-pivaloyloxymethyl-5-fluorouracil (1-pivaloyoxymethyl-5-FU) have been designed as prodrugs to enhance the bioavailability of 5-FU by their increased lipophilicity (log P values of 0.38 and 0.66) and increased aqueous solubility (12.2 and 11.4 mg/mL) as a result of decreased hydrogen bonding due to the blocking of 1-NH group. Comparative cumulative permeation studies showed that the 1-

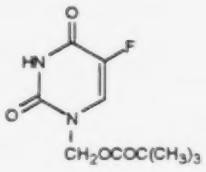
butyryloxymethyl-5-FU penetrates the skin at a rate about 5 times faster than 5-FU, while the 1-pivaloyoxymethyl derivative is absorbed at a rate about 2 times faster. The observation that the 1-pivaloyoxymethyl-5-FU, which has a higher partition coefficient,



5-FU



1-butyryloxymethyl-5-FU



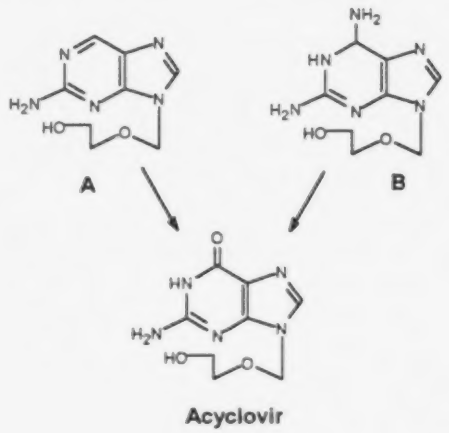
1-pivaloyoxymethyl-5-FU

showed a lower rate of penetration than the 1-butyryloxymethyl-5-FU suggests that factors other than lipophilicity are important.

1.5.1.3. Reductive or oxidative processes.

In some cases, the conversion from a prodrug to its parent drug involves biochemical reductive or oxidative processes.

Acyclovir is a clinically useful antiherpetic agent. However, it has a poor oral bioavailability (10-20%) in humans due to its poor water-solubility and lipophilicity which are due to intermolecular hydrogen bonding formed between the C=O and NH moieties.[223-225] In an attempt to improve the oral bioavailability, 6-deoxy (A) and 6-deoxy-6-amino (B) derivatives of acyclovir have been studied as prodrugs to acyclovir with an increased solubility in both water and organic solvents due to the absence of a C=O moiety.[226,227] These prodrugs are



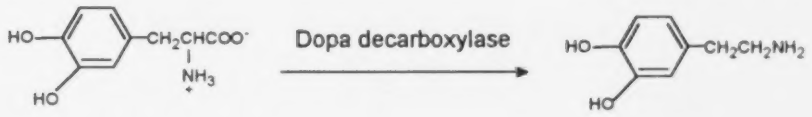
A **B**

Acyclovir

converted to acyclovir by adenosine deaminase and xanthine oxidase, respectively.[228] However, 6-deoxy-6-amino-acyclovir showed only a modest increase in acyclovir plasma levels in dogs and rats,[226] whereas 6-deoxy-acyclovir showed a 5-6 fold increase in bioavailability relative to acyclovir in rats and human.[228,229]

1.5.1.4. Decarboxylation.

Parkinson's disease is characterized by a dopamine deficiency in brain. However, dopamine administration as a direct therapy in treating this disorder is ineffective since it does not cross the BBB due to its polarity and highly ionized state at physiological pH. L-Dopa has been developed to overcome this problem, since it can be absorbed from the GI tract prior to entry into the CNS via the active transport system for amino acids since L-dopa is an amino acid.[230] In the CNS, L-dopa is converted to dopamine by dopa decarboxylase. Therefore L-dopa is a prodrug which converts to the parent drug via a decarboxylation process.



L-Dopa **Dopamine**

1.5.1.5. Regeneration of a double bond via an elimination reaction.

5,6-Disubstituted-5,6-dihydropyrimidines represent another example of prodrugs requiring a chemical or enzymatic reaction, which may be a thiol-mediated process, to be converted to their corresponding parent drugs. It has been shown that 5-bromo-5-fluoro-6-methoxy-5,6-dihydro-2'-deoxyuridine acts as a slow releaser of 5-fluoro-2'-deoxyuridine *in vivo*.[231] More recently, it has been reported that 5-halo-6-methoxy-5,6-dihydro

derivatives of 5-ethyl-2'-deoxyuridine (EDU) exhibit improved pharmacokinetic parameters, including increased brain uptake and a prolonged blood residence time relative to EDU.[232,233] EDU exhibits antiviral activity against herpes simplex virus type-1 (HSV-1), type-2 (HSV-2) and vaccinia virus. However, it undergoes rapid metabolism to the inactive 5-ethyluracil in the presence of pyrimidine phosphorylases. To prevent this undesired glycosidic bond cleavage, 5-halo-6-methoxy-5,6-dihydro-EDUs, which are not substrates for pyrimidine phosphorylase, were designed as prodrugs. It has been demonstrated that 5-bromo-6-methoxy-5,6-dihydro-EDUs which are rapidly converted *in vivo* to EDU results in an elevated concentration of EDU in blood relative to when EDU is administered in mice by i.v. injection. 5-Chloro-6-methoxy-5,6-dihydro-EDUs are converted to EDU at a slower rate.[232]

5-Halo-6-methoxy-5,6-dihydro-EDU **EDU**

1.5.2. The application of the prodrug approach to nucleosides.

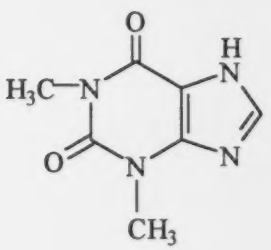
Most nucleoside analogs as such are inactive and they represent another class of prodrugs. Like AZT discussed earlier, they all need to be biotransformed to their triphosphate in order to exert certain biological functions.

Nucleoside ⟶ Nucleoside_MP ⟶ Nucleoside_DP ⟶ **Nucleoside_TP (active)**

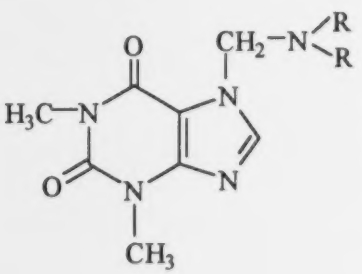
The prodrug approach has been successfully applied to a number of nucleoside analogs to improve their utility and efficacy.

1.5.2.1. Solubility. Most nucleosides have a low water solubility and low partition coefficient (P). Modification, by introducing a water- or lipid-soluble moiety, will increase the water or lipid solubility of the drug. If this new compound converts to the original nucleoside *in vivo*, then this modified compound can be considered to be a prodrug.[234] It is known that intermolecular hydrogen bonding decreases drug solubility in both water and organic solvents. Therefore, modification to reduce intermolecular hydrogen bonding of parent drug by blocking or removing the moiety involved in the formation of the intermolecular hydrogen bonding usually increases both water and lipid solubilities of the parent drug.

Theophylline, for example, has poor solubilities in both water and organic solvents due to intermolecular hydrogen bonding caused by the presence of NH_2 and C=O moieties. N-Mannich bases of theophylline have been prepared as prodrugs to improve



Theophylline



N-Mannich bases of theophylline

the lipophilicity for effective delivery of theophylline through skin.[235] It was found that these prodrugs have a significantly increased water solubility and lipophilicity relative to theophylline, as shown in Table 1.5.1. This effect is a result of the absence of

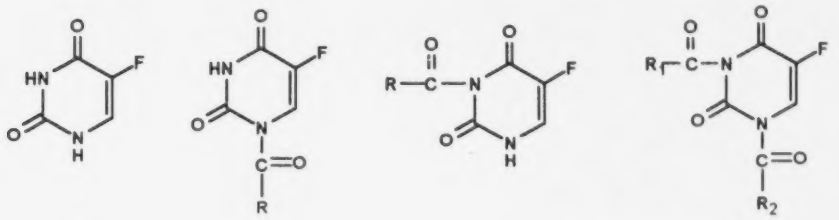
intermolecular hydrogen bonding from the N-Mannich bases of theophylline due to the absence of NH_2.

Table 1.5.1. Solubilities of theophylline and its N-Mannich base prodrugs.[235]

Compound	Solubility (mg/mL)	
	water	isopropylmyristate
Theophylline	8.3	0.095
R = methyl	100	7.6
R = ethyl	100	32.5
R = propyl	100	27.6
R = morpholino	100	1.3
R = pyrrolidino	100	10.7

1.5.2.2. Bioavailability. (absorption and distribution). If an active drug does not have optimal water or lipid solubility for absorption or distribution to the site of action, a prodrug with the proper property may increase its absorption or delivery. Ideally the prodrug should be converted to the active drug after it reaches its site of action.[236-239] The potential usefulness of the prodrug approach to enhance bioavailability of an active agent can be illustrated with 5-FU and its N-acylated prodrugs. To improve the oral and rectal absorption of 5-FU, enhancement of lipophilicity by the prodrug approach was pursued. The octanol/water partition coefficient of 5-FU is only 0.15 and this low lipophilicity may be a predominant factor for the poor biomembrane permeability of the drug. Various N^1-acyl, N^3-acyl and N^1, N^3-diacyl derivatives of 5-FU were shown to be readily hydrolyzed in human plasma.[240-242] Although N^1-acyl derivatives proved to be very unstable, N^3-acyl derivatives offered a promising approach to obtain prodrugs to 5-FU. These prodrugs are more lipophilic than 5-FU and some are also more soluble in water relative to 5-FU as shown in Table 1.5.2. As mentioned earlier, this apparently

anomalous behavior can be attributed to decreased intermolecular hydrogen bonding in the crystal lattice achieved by blocking the 3-NH group.



5-FU **N^1-acyl-5-FU** **N^3-acyl-5-FU** **N^1, N^3-diacyl-5-FU**

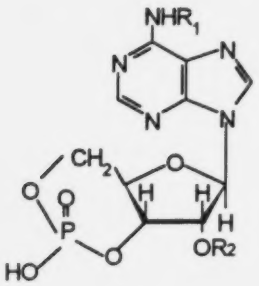
Table 1.5.2. Solubilities of 5-FU and its N^3-acyl prodrugs.[242]

Compound	Solubility in buffer (mg/mL)	log P
5-FU	11.1	-0.83
3-Acetyl-5-FU	42.8	-0.34
3-Propionyl-5-FU	35.3	0.19
3-Butyryl-5-FU	ND	0.67
3-Benzoyl-5-FU	1.3	0.80

ND = not determined.

1.5.2.3. Stability. An active drug may also undergo rapid metabolism to an inactive metabolite. A prodrug can therefore be designed to mask or block the moiety which undergoes the unwanted metabolism. The conversion of the prodrug to drug should take place after it has reached its site of action.[243-246] Cyclic 3',5'-adenosine monophosphate, a polar nucleotide regulator of glycogenolysis, has been acylated. Thus, N^6-2'-O-dibutanoyl, N^6-butanoyl and N^6-octanoyl derivatives of 3',5'-adenosine monophosphate have shown superior and prolonged hyperglycemic activity compared to 3',5'-adenosine

monophosphate.[247] The author attributed this greater activity to increased entrance into cells and/or the resistance of the derivatives to inactivation by phosphodiesterases.



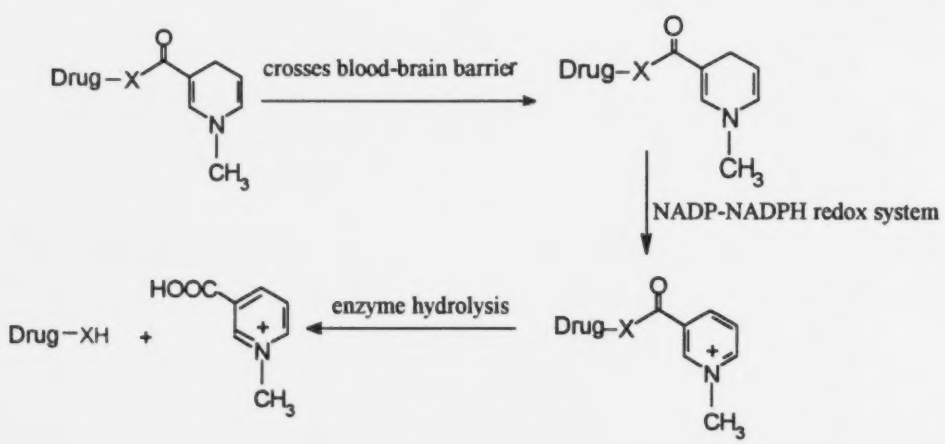
R_1=R_2=H, Cyclic 3',5'-adenosine monophosphate

R_2=H, R_1=acyl group

R_1=H, R_2=acyl group

R_1=R_2=acyl group

1.5.2.4. Specific-site delivery. To achieve a specific-site delivery, the prodrug either should have a specific localization in the target site or be converted to the active drug at the target site by unique enzymes present there. In 1981, a redox chemical drug delivery system consisting of a dihydropyridine-pyridinium salt was proposed by Bodor *et al.* to target active drug to the brain.[248] This system is based on redox chemistry, in which a biologically active compound linked to a lipophilic dihydropyridine carrier can readily



XH = OH, NH_2

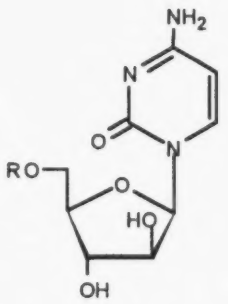
penetrate the BBB because of the enhanced lipophilicity. Oxidation of the carrier 1,4-dihydropyridine moiety to the hydrophilic pyridinium salt (quaternized salt) in the brain slows its elimination due to a "lock-in" mechanism of polar compounds in the brain. Subsequent cleavage of the quaternary carrier drug results in a sustained production of the drug in the brain. This method has been explored as a general strategy to target drugs to the brain as reported for the brain-specific delivery of dopamine, phenylethylamine, N-pyridinium-2-carbaldoxime chloride, testosterone, estradiol, tryptamine and other pharmacological agents.[249-252] This approach has also been applied to nucleoside analogs in order to enhance the brain uptake of the nucleoside analogs.[253-258]

Specific-site delivery can also be illustrated by the selective antiviral action of acyclovir. Acyclovir itself is inactive and it is a prodrug with specific-site delivery. Its antiviral function is due to the triphosphate of acyclovir and its selectivity toward virus relies on its specific phosphorylation by viral thymidine kinase and specific inhibition of viral polymerases. Acyclovir is selectively phosphorylated by a viral-specific thymidine kinase to the corresponding monophosphate. Subsequent conversion of the monophosphate to diphosphate is catalyzed by guanylate kinase;[259] the diphosphate is then converted to the triphosphate.[260] In contrast, uninfected cells do not phosphorylate acyclovir. Furthermore, selective toxicity to infected cells is derived from the fact that acyclovir triphosphate selectively interacts with viral α-DNA polymerases as its structure resembles that of the essential DNA precursor, deoxyguanosine triphosphate. However, it is not a substrate for the normal cellular polymerase. Therefore acyclovir (prodrug to acyclovir triphosphate) exhibits a high degree of selective toxicity against viral-infected cells.

1.5.2.5. Sustained release. It is crucial to maintain a steady concentration of drug over a long period of time for drugs with narrow therapeutic indices. A prodrug with slow

conversion to active drug will give a sustained release of the active drug. There are many advantages of slow and prolonged release of active drug: a) It reduces dosing frequency. b) It eliminates nighttime administration. c) It eliminates toxicity caused by concentration peaks produced from administration of a parent drug and subtherapeutic levels as a result of fast clearance of the parent drug.

To maintain an effective blood level of the very short half-lived drug cytosine arabinoside (an immunosuppressive, antiviral and cytotoxic agent), long term i.v. infusions of considerable inconvenience to the patient and nursing staff are needed. 5'-Acyl derivatives of cytosine arabinoside as well as 2'- and 3'- esters have been synthesized and tested as prodrugs to provide a slow release of cytosine arabinoside.[261-265] A variety of 5'-acylated derivatives were found to be more potent than their parent drug (cytosine arabinoside) in suppressing the immune response in mice, hamsters and rats, and as antitumor agents in mice after intraperitoneal injection. After injection of these 5'-acylated derivatives to mice, there is a finite time required for dispersion and solubilization. The freely circulating derivatives are resistant to deamination (inactivation). After enzymatic hydrolysis to the free drug, the latter is then free to exert its inhibitory activities. The net effect is the maintenance of relatively low levels of the active drug for long periods of time.



R = acyl

1.5.2.6. Toxicity. A drug may exhibit toxicity if it accumulates selectively in certain tissues or organs and interacts with some receptor in the organ. Any drug-receptor interaction will be dependent on the amount of drug reaching the receptor and the way that it binds to the receptor. The toxicity of an active drug at a non-target site can be minimized by using prodrugs which localize only at the site of action (specific-site

delivery) or only convert to active drug at the site of action. In this regard, the selective toxicity of acyclovir toward viral-infected cells is a result of specific conversion of acyclovir to its active form, i. e., acyclovir triphosphate, by the target cells (infected cells). Noninfected cells are unable to biotransform the drug to the triphosphate due to the lack of virus-specific thymidine kinase.

1.5.3. Prodrugs to nucleoside RT inhibitors.

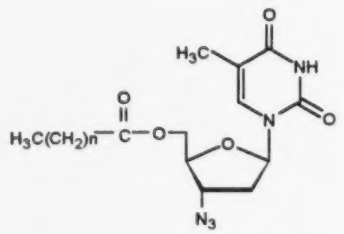
As part of the development strategy for AIDS therapy, many studies have developed a variety of prodrugs as potential RT inhibitors in order to improve their brain uptake, limit their bone marrow toxicity, sustain their release from the drug formulation or target them to macrophages, which could be a reservoir of HIV infection.[205-209,253,257,258,266]

As mentioned earlier, serious neurologic dysfunction, i.e., the AIDS dementia complex, is an important cause of morbidity in many patients.[12,13] Brain macrophages, microglia and multinucleated cells can be productively infected with HIV-1.[127-130,267-269] Proviral DNA, viral nucleic acid, viral antigens, and HIV-1 virions are present in the brain of patients with AIDS dementia.[11-13] The invasion of the nervous system by HIV-1, coupled with the possibility that it could create a reservoir of persistent infection even if peripheral clearance were realized,[270] has led to the general agreement that an ideal chemotherapeutic agent for HIV should penetrate and be highly active in the CNS. Most nucleoside analogs, being polar molecules, do not readily cross the BBB. For example, AZT and other nucleoside RT inhibitors have been found not to penetrate through the BBB efficiently.[134,135,164,165] The ability of a compound to traverse the BBB and enter the brain is dependent, at least in part, on its lipid solubility. The application of the lipophilic prodrug concept to the design of prodrugs has attracted a lot of attention to improve *in vivo* biodistribution of AZT and other RT inhibitors.

Fast clearance of AZT and other ddNs from blood presented a dosing problem, which resulted in a limited beneficial outcome clinically. Maintenance of optimal therapeutic concentrations with oral intermittent dosage regimens is difficult because of the short half-life and dose-limiting toxicity. Continuous intravenous infusion has been shown to be superior to intermittent bolus,[89] but it is technically complicated and inconvenient. Moreover, toxicity is expected to be reduced by controlled release of the active agent from a prodrug. Esterification of the 5'-OH of AZT and other ddNs RT inhibitors, as well as modifications at nucleobases, has been the most common approach including the following modifications.

1.5.3.1. Aliphatic esters.

Aliphatic ester prodrugs to AZT were designed to improve the retention of AZT in the body by slow release of AZT from the prodrugs (structure is shown in Figure 1.5.1.).[205] The prodrugs are generally much more lipophilic than AZT (log P values in chloroform/phosphate buffer ranged from 1.61 to > 5 vs. -0.31 for AZT). The partition coefficients of the esters increase as the acyl chain is lengthened. All these esters are susceptible to acidic and basic hydrolysis, but they are stable under moderate pH conditions (pH 4.0 and 7.0). The enzymatic hydrolysis rate in the presence of different mouse enzyme preparations, including plasma, liver, kidney, and intestine, has been demonstrated to be dependent on the length of the acyl chain. Initially, the rate increases with an increase in the chain length (C10-AZT showed



n = 0-16

Figure 1.5.1. The general chemical structure of 5'-aliphatic acid ester prodrugs of AZT.

the highest hydrolysis rate) and then decreases with a further increase in the acyl chain length. AZT and three of the prodrugs (acetate, caprate, and stearate) were administered to mice intraperitoneally, and plasma concentrations of AZT were measured. The AZT concentrations in plasma following the acetate administration rapidly decreased with a half-life ($t_{1/2}$) of 14.5 min which is similar to that demonstrated in direct AZT administration ($t_{1/2}$ = 11.7 min). However, the AZT concentrations following the caprate or stearate administration decreased slowly and were maintained for as long as 4 h after dosing (2 h after AZT or AZT acetate). The results suggest that the AZT concentrations following administration of the aliphatic ester prodrugs are dependent on the length of the acyl chain, which alters the rate of hydrolysis and elimination from the body, and an optimal sustained release of AZT may be obtained by selecting an appropriate acyl group.

1.5.3.2. Amino acid esters.

A variety of 5'-esters of AZT have been prepared to improve the biotransport across membranes and to overcome the problem of rapid elimination of AZT. Among the esters reported, amino acid (tyrosinyl, isoleucinyl, lysinyl, phenylalanyl, glutamyl) esters of AZT were synthesized in an attempt to deliver AZT more efficiently by an active transport system used for amino acids to target cells at higher concentrations than that which can be achieved by AZT alone.[206] Moreover, bone marrow progenitor cells may lack the amino acid active transport system,[271] which may lead to a reduced toxicity. It was found that phenylalanyl- and isoleucinyl-AZT are more lipophilic, with P values of 3.3 and 10.3, respectively, whereas the remaining amino acid prodrugs studied are less lipophilic (P values ranged from 0.01 to 0.94). Tyrosinyl- and lysinyl- derivatives

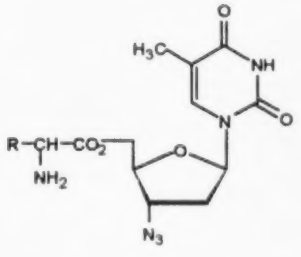


Figure 1.5.2. The general structure of 5'-amino acid prodrugs.

of AZT were shown to be unstable upon incubation with phosphate buffer at 37°C. All of the amino acid derivatives were found to be susceptible to the action of plasma esterases, with $t_{1/2}$ values in the range of 10 to 120 min, except for the isoleucinyl derivative which has an increased stability toward hydrolysis, with a $t_{1/2}$ greater than 240 min. The $t_{1/2}$ of the prodrugs in rat hepatic microsomes varied from 5 to 30 min, indicating that the microsomal enzymatic hydrolysis was relatively rapid. Cell membrane permeability of AZT and these prodrugs was measured in H9 cells. It was found that only the isoleucinyl-derivative, which has the highest partition coefficient, resulted in greater permeation than AZT (approximately 50% higher) at short time intervals. The other amino acid derivatives had decreased cellular uptake, particularly the lysinyl- and glutamyl-derivatives, which also have significantly lower partition coefficients, suggesting that lipophilicity plays an important role in the permeation of the prodrugs. The importance of an active transport system for these amino acid derivatives in the permeation of these prodrugs is not understood.

1.5.3.3. Phosphodi- or tri-esters.

Like AZT, all ddNs must be phosphorylated intracellularly to their 5'-triphosphate derivatives by nucleoside kinases to interact with their target enzyme, HIV RT to exert their anti-HIV activity. In many cases, the ddNs have a poor affinity for nucleoside kinases, e.g., ddC for deoxycytidine kinase, d4T, 3'-deoxythymidine (3'-dT) and 2',3'-dideoxyuridine (ddU) for thymidine kinase, 2',3'-dideoxyadenosine for adenosine kinase and deoxycytidine kinase, and 2',3'-dideoxyinosine (ddI) for 5'-nucleotidase.[272-276] It has also been found that the dependence on phosphorylation for activation of the particular nucleoside analog may be a problem in cells where the nucleoside kinase activity is known to be low or absent (monocyte/macrophage).[277] One of the possibilities for improving the efficacy of a ddN would be to bypass at least in part these phosphorylation steps and to

administer the preformed phosphorylated derivatives. Unfortunately phosphorylated derivatives, due to their increased hydrophilicity, are not likely to cross membranes efficiently and thus to reach their intracellular targets. Therefore prodrugs of ddN mono- or di-phosphate, in which the phosphate groups were modified by hydrophobic moieties, have been widely studied for delivering the phosphorylated ddN into targeted cells. Moreover, phosphorylated ddNs have been studied as lipophilic prodrugs to enhance the efficacy of anti-HIV agents in brain.[278]

Glycosyl phosphotriesters were proposed as lipophilic prodrugs to AZT to increase the brain uptake (structure shown in Figure 1.5.3.).[278,279] This system presents additional advantages over the esters discussed earlier (aliphatic and amino acid esters): (i) the hydrolysis product is the 5'-monophosphate which is the first metabolite of AZT anabolism by cellular kinases. This can be very important for macrophages which have lower levels of kinases and for reducing the toxicity associated with AZT because the thymidine kinase is not needed. (ii) the degradation products, a carbohydrate (glucose or mannose) and the lipidic alcohol (hexadecanol), do not contribute to toxicity. (iii) with increased lipophilicity expected because of the hexadecyl group, the solubility of the prodrugs in water due to the presence of glycosyl moiety allows oral administration.[278] The evaluation studies suggested that the glycosyl phosphotriesters resulted in a significantly improved pharmacokinetic profile of AZT-5'-phosphate in brain compared to that after AZT (peak concentration 156 nmol/g vs. 5 nmol/g; half-life 24 h vs. 1 h; AUC 4366 nmol h/g vs. 4 nmol h/g).[278]

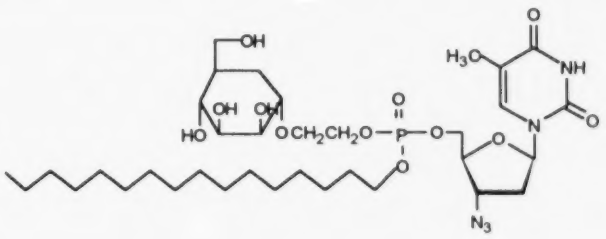
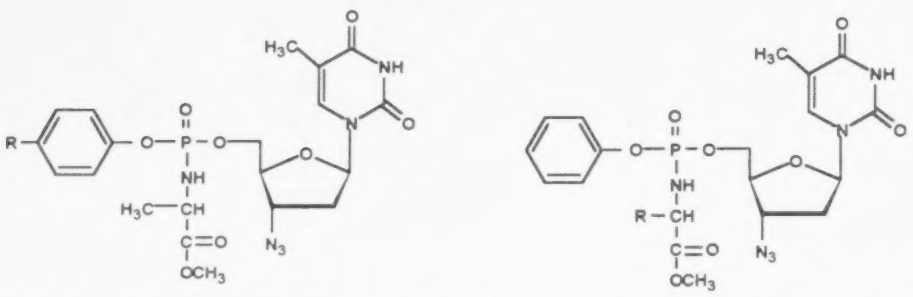


Figure 1.5.3. The chemical structure of hexadecyl 2-(α-D-mannopyranosidyl)ethyl-3'-azido-3'-deoxy-5'-thymidinyl phosphate.

Macrophages may be resistant to AZT because of insufficient phosphorylation,[96] due to the lower levels of nucleoside kinases present there. Aryl phosphate derivatives[280] (structures are shown in Figure 1.5.4.) and phospholipid analogs of AZT[208] (structures are shown in Figure 1.5.5.) were proposed as prodrugs of AZT-triphosphate to overcome the problem of lack of thymidine kinase in certain cell types. *In vitro* evaluation of aryl phosphate derivatives of AZT revealed that these compounds have a pronounced and selective anti-HIV activity. Some of these compounds retain marked antiviral activity in thymidine kinase-deficient mutant CEM cells in which AZT was virtually inactive.[280]



R = H, CH_3, C_2H_5, n-C_3H_7, n-Pentyl, CH_3O, F

R = H, CH_3, i-C_3H_7, CH_2Ph

Figure 1.5.4. Aryl derivatives of AZT monophosphate.

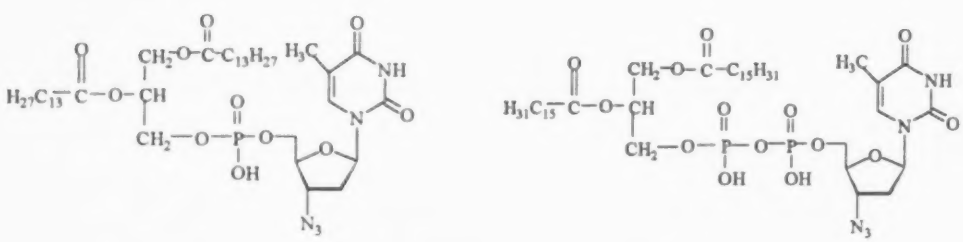


Figure. 1.5.5. The chemical structures of phospholipid prodrugs to AZT.

The idea of Hostetler *et al.* to target the prodrug to macrophages by incorporation of these lipophilic prodrugs into liposomes has resulted in an enhanced uptake by the macrophages.[208] The product released in macrophages is AZT-5'-phosphate, which bypasses part of the activation step and enhances the activity of AZT. More recently, new lipophilic alkyl/acyl dinucleoside phosphate derivatives of AZT have been synthesized (structures shown in Figure 1.5.6.). The liposomal preparations of these derivatives allow treatment regimens with intermittent and delayed application schedules. AZT was significantly less effective in these treatment regimens. The liposomal preparation of these newly synthesized dinucleoside phosphate derivatives have shown superior *in vivo* activity in the Rauscher leukemia model. Furthermore, the particular nature of the liposomes may lead to higher drug levels in plasma and in virus-infected phagocytosing cells such as macrophages, monocytes and lymphocytes due to the natural affinity of liposomes for cells of the mononuclear phagocyte system.

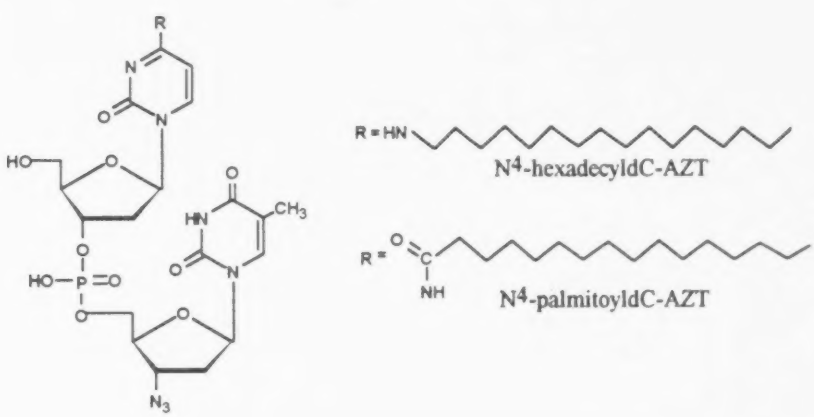


Figure 1.5.6. The chemical structures of alkyl/acyl dinucleoside phosphate derivatives of AZT. N^4-hexadecyl-2'-deoxyribocytidylyl-(3'-5')-3'-azido-2',3'-deoxythymidine (N^4-hexadecyldC-AZT) and N^4-palmitoyl-2'-deoxyribocytidylyl-(3'-5')-3'-azido-2',3'-deoxythymidine (N^4-palmitoyldC-AZT).

ddU is ineffective in HIV-infected human T cells because it is not biotransformed to the active metabolite ddU-5'-triphosphate. This problem cannot be overcome by supplying the preformed nucleotides, since these compounds are unable to penetrate cell membranes due to their poor lipophilicity. Bis(pivaloyloxymethyl)-2',3'-dideoxyuridine-5'-monophosphate was designed as lipophilic prodrug of ddU-5'-monophosphate to penetrate cell membranes followed by the release of ddU-5'-monophosphate as illustrated in Figure 1.5.7.

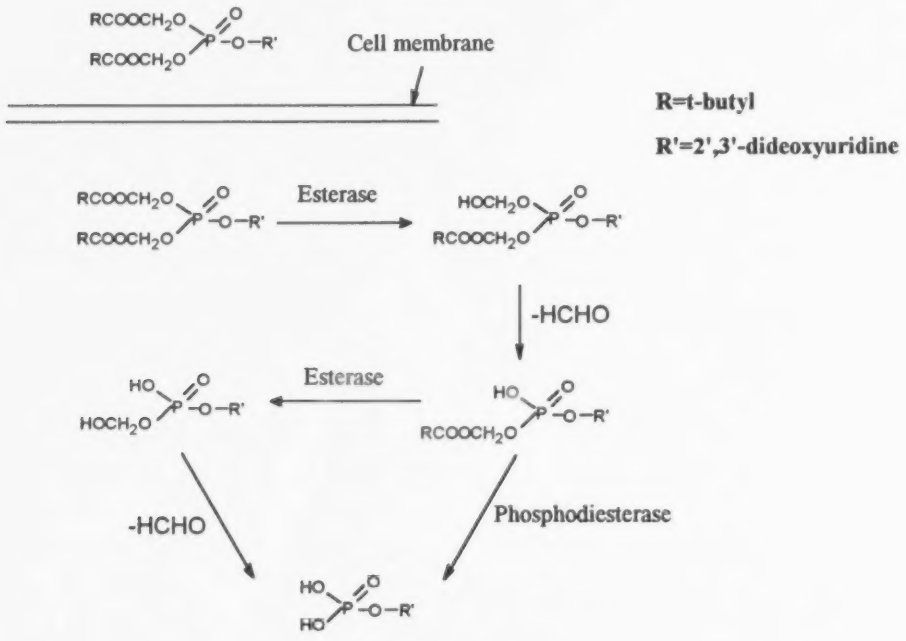


Figure 1.5.7. Diagram of penetration and metabolism of Bis(pivaloyloxy-methyl)-2',3'-dideoxyuridine-5'-monophosphate.

Furman *et al.*[93] have demonstrated that AZT-MP accumulation causes a severe inhibition of cellular dTMP kinase which catalyzes the conversion of AZT-MP to AZT-

DP, resulting in a blockade of further phosphorylation steps. On the contrary, d4T appeared unable to induce the same effect[281] and is clearly unable to inhibit its own phosphorylation.[282,283] For these reasons, it was clear that increasing the concentration of AZT-MP was of little value. d4T, however, was a much better candidate, as d4T-MP does not accumulate because it is readily converted to the triphosphate derivative. Several 5'-monophosphate d4T derivatives and d4T-MP-d4T derivatives have been synthesized as potential lipophilic prodrugs of d4T (structures shown in Figure 1.5.8.).[284]

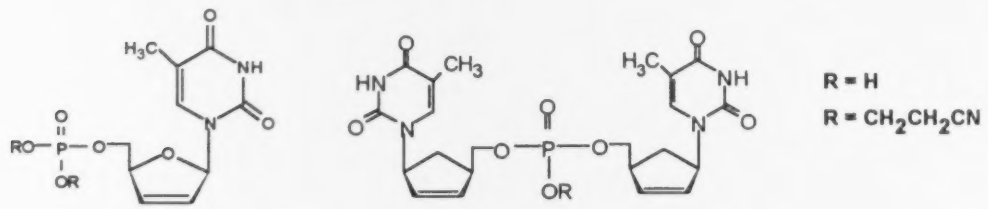


Figure 1.5.8. The chemical structures of selected phosphoester prodrugs to d4T.

In the case of 3dT which is weakly active in HIV-infected cells because of its slow phosphorylation by cellular thymidine kinase, its diphosphate dimyristoylglycerol (3'-deoxythymidine-5'-diphosphate-3-*sn*-1,2-dimyristoylglycerol, 3dTDP-DMG), a phospho-lipid prodrug, was synthesized and found to be 18 to 50 fold more effective than 3dT *in vitro*. In thymidine kinase-deficient cells infected with HIV, 3dTDP-DMG exhibited substantial activity whereas 3dT and AZT were virtually inactive.[285] The increased efficacy is at least partly due to bypass of thymidine kinase.

ddC was found to have a half-life of 1.2 h in human plasma. To achieve an antiretroviral effect, patients received 30 to 90 μg/kg of ddC every 4 h. This dose was found to cause toxic effects, in particular neuropathy.[286] Encapsulation of ddC in erythrocytes was proposed to achieve a slow release of ddC at effective concentrations but below toxic concentrations. However, ddC was found to escape from the

erythrocytes, and encapsulation of the ddC monophosphate (ddC-MP, prodrug to ddC) was studied. When erythrocyte-encapsulated ddC-MP was incubated with a physiological saline solution, the appearance of ddC in the medium was observed, which is a result of hydrolysis of ddC-MP by human erythrocyte lysates followed by the release of ddC from the cells.

1.5.3.4. 1,4-Dihydro-1-methyl-3-[(pyridylcarbonyl)oxy] ester.

As discussed earlier, 1,4-dihydro-1-methyl-3-[(pyridylcarbonyl)oxy] (DP) ester has been proposed and widely used to provide a brain-specific delivery of a variety of compounds.[218,248-251,287] 5'-DP derivatives of AZT, d4T, ddC and 3'-azido-2',3'-dideoxyuridine (AZdU) have been synthesized to utilize this system to achieve higher concentrations of the individual nucleoside analog in brain (example is shown in Figure 1.5.9.).[253,257,258,289-293]

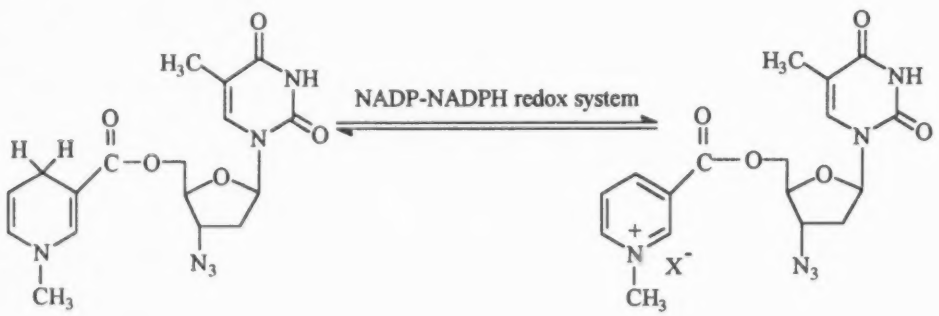


Figure 1.5.9. Dihydropyridine-pyridinium salt chemical delivery system.

In vivo studies indicated that significantly greater amounts of AZT, d4T, and AZdU could be delivered to the brain following administration of prodrug than the parent drugs.[257,258,289-291,293] Evaluation of the AZT prodrug[291] demonstrated increased cellular uptake (about 50% higher in comparison to AZT) in H9 cells and peripheral blood

lymphocytes (PBL), due to its higher lipophilicity (P = 4.8 relative to 1.2 for AZT). A three-fold higher concentration of AZT in rat brain and a two-fold higher concentration in the CSF of dogs was achieved, compared to an equimolar dose of AZT.[257,292] An increased brain uptake was also observed in a rabbit study.[294] However, constant lower concentrations of AZT (50% reduction) were observed in plasma after DP-AZT compared to those after AZT. It is not clear if this reduction will cause subtherapeutic concentrations in human when equimolar doses of the prodrug are administered. DP-d4T resulted in a sustained release of d4T in the brain of mice, with a half-life of 25 h.[258] *In vivo* studies of DP-AZdU in mice demonstrated a significantly increased brain concentration and brain half-life of AZdU (4.34 vs 0.84 h) following prodrug administration relative to AZdU administration.[257]

However, the same degree of improvement was not observed by using DP-ddC. ddC is one of the most potent ddN analogs *in vitro*, but it is much less effective than is AZT with respect to penetration of the CNS.[295-298] The utility of the dihydropyridine chemical delivery system to enhance the brain uptake of ddC was evaluated with 5',N^4-bis-[(1,4-dihydro-1-methyl-3-pyridinyl)carbonyl)]-2',3'-dideoxy-cytidine. Unfortunately, no significant increase in ddC concentrations in the brain after dosing with the disubstituted redox prodrug, relative to dosing with ddC, was observed. This study emphasizes the importance of drug selection when attempting to utilize the dihydropyridine chemical delivery system to augment anti-AIDS drug delivery to the brain.

The major limitation of the approach was found to be facile oxidation of the DP-modified prodrugs by oxygen in air as well as in solution, which made even iv formulation difficult.

1.5.3.5. Phosphate esters of ether lipid analogs.

This innovative approach has combined two active anti-HIV moieties, i.e., nucleoside analog and ether lipid analogs (ELs) which are active and have potent anti-HIV activity, into one molecule. The two species released act at different stages of the viral replication. The ELs are membrane interactive and the mechanisms of their action involve a shift in virus assembly from the plasma membrane to intracytoplasmic vacuoles and production of defective virus,[299] whereas the nucleoside analog targets the RT. The advantages of this design are: 1) the prodrug is more lipophilic than the parent because the ELs are lipophilic compounds that accumulate in the plasma membrane of T-lymphocytes and monocytes/macrophages and undoubtedly cross the BBB in the host; 2) the ELs are synergistic with AZT and resulted in an enhanced efficacy; 3) the two molecules have different mechanisms of action and the ELs are not associated with bone marrow toxicity observed with AZT, therefore lower toxicity would be expected; 4) the development of viral resistance to two active drugs most likely would occur at a slower rate than to either compound alone; and 5) the nucleoside analog is released as its monophosphate, potentially decreasing the toxicity since the thymidine kinase pathway may not be needed. The results demonstrated that AZT and the selected EL analog, AM-18-OEt, produced an apparent synergistic activity against HIV.[207] Conjugates of amidoalkyl, oxyalkyl, and thioalkyl ELs with AZT and ddI were synthesized (structures shown in Figure 1.5.10.). It is reasonable to assume that EL-AZT conjugates may provide a slow release of AZT-MP and AZT into treated cells due to metabolism by cellular phosphatases. In infected monocyte/macrophage cells, as expected, the selected conjugate AZT-AM-18-OEt showed increased activity (3 fold) relative to AZT due to direct action of the conjugate or to the release of AZT-MP upon hydrolysis of the conjugate, therefore diminishing the resistance associated with decreased kinase activity in this cell line.

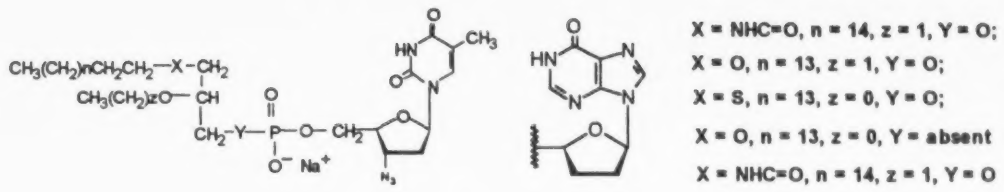


Figure 1.5.10. The chemical structure of EL prodrugs of AZT and ddI.

1.5.3.6. N^4-substituted-cytidine derivatives.

Enhancement of lipophilicity has been a common approach for improving uptake of drugs into brain. In many cases, lipophilicity was increased at the expense of water solubility. However, the primary target cells of HIV are most abundant in the circulation and drug availability for cellular uptake and tissue distribution is a function of the unbound drug concentration in the plasma. Therefore, water solubility is an important factor. N^4-substituted-cytidine derivatives have been studied as more water-soluble lipophilic prodrugs. ddC and 3'-fluoro-2',3'-dideoxycytidine (3'-F-ddC) are anti-HIV candidates that have poor solubilities in both water and organic solvents due to intermolecular hydrogen bonding caused by the NH_2 and C=O moieties. Modifications on the exocyclic amino group of the nucleosides (ddC and 3'-F-ddC) were expected to increase both lipophilicity and water solubility of resultant compounds due to modification of the NH_2 group which contributes to intermolecular hydrogen bonding. A number of N^4-substituted ddC (1a-1g) and 3-F'-ddC (2a-2g) derivatives (Figure 1.5.11.) have been synthesized as prodrugs in an attempt to increase lipophilicity without a decrease in water solubility.[300] These prodrugs have been shown to have an increased lipophilicity as shown in Table 1.5.3. The estimated water solubility of the most stable diisopropyl derivatives (1d,2d) at room temperature is increased relative to their corresponding parent compounds.

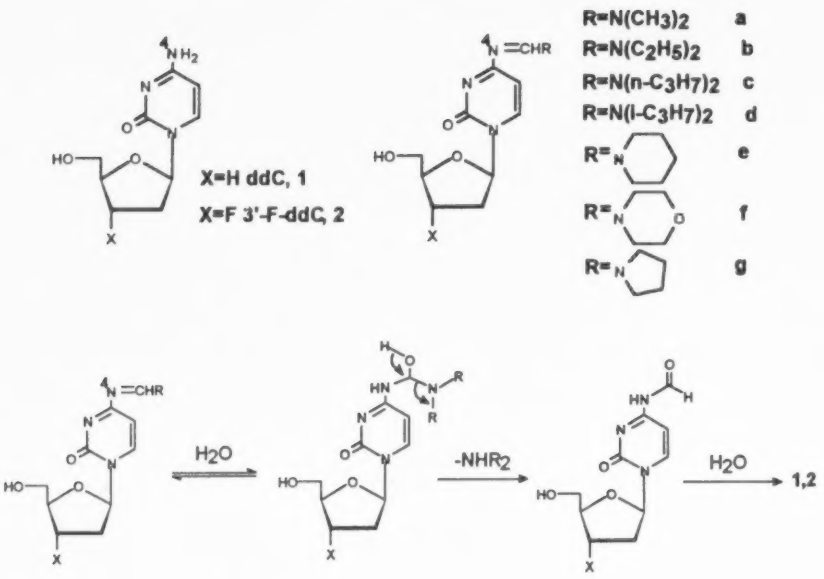


Figure 1.5.11. The chemical structures of N^4-substituted-cytidine derivatives and conversion to their parent drugs.

Table 1.5.3. The partition coefficients and water solubilities of ddC, 3-F'-ddC and their N^4-substituted derivatives.

Compound code	$P_{1\text{-octanol/PBS}}$	water solubility (mg/mL)	Compound code	$P_{1\text{-octanol/PBS}}$	water solubility (mg/mL)
ddC	0.073	~78	3-F-ddC	0.128	~15
1a	0.112		2a	0.229	
1b	0.873		2b	1.688	
1c	8.87		2c	22.38	
1d	5.30	> 300	2d	13.80	~25
1e	1.14		2e	2.28	
1f	0.108		2f	0.211	
1g	0.341		2g	0.688	

1.5.3.7. Others.

Although ddI has been approved as an anti-HIV agent for the treatment of patients who cannot tolerate AZT or who develop resistance to AZT, its lack of stability in acidic media upon oral administration coupled with side effects such as pancreatitis and peripheral neuropathy limit its usefulness as an orally active anti-HIV drug. Moreover ddI does not cross the BBB in significant amounts.[155] In order to increase the stability, 2'-fluoro-2',3'-dideoxy-2'-arabinofuranosylinosine (2'-F-ara-ddI) was synthesized. It is as potent as ddI, and is undergoing clinical trials as an anti-HIV agent. To enhance the brain uptake, 2'-fluoro-2',3'-dideoxy-2-arabinofuranosylpurine (2'-F-ara-ddP) was designed as a more lipophilic prodrug of 2'-F-ara-ddI,[301] which is presumably converted to the parent drug by utilizing an *in vivo* biotransformation system such as xanthine oxidase that is known to be present in brain (Figure 1.5.12.).[302] It was found that 2'-F-ara-ddP was completely converted to 2'-F-ara-ddI by mouse liver homogenate within 20 min, which suggested that extensive enzymatic oxidation had occurred. The role of xanthine oxidase in the conversion of 2'-F-ara-ddP to 2'-F-ara-ddI was confirmed by conducting an *in vitro* study in which the conversion was examined in the presence or absence of the xanthine oxidase inhibitor, allopurinol; the half-lives of 2'-F-ara-ddP were found to be 7.4 and 3.4 h, respectively, indicating that the conversion was catalyzed by xanthine oxidase. This

2'-F-ara-ddP **2'-F-ara-ddI**

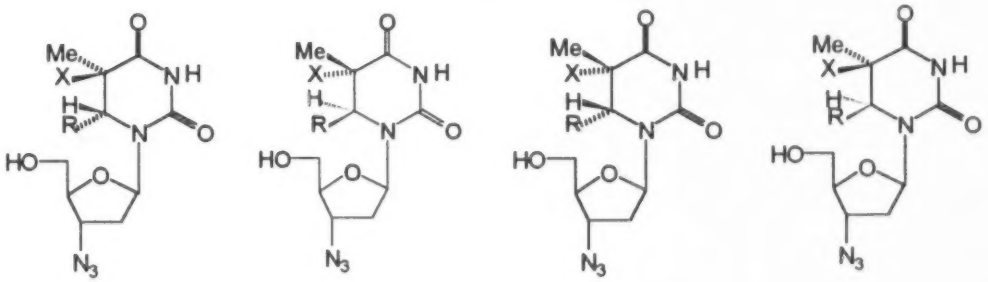
Figure 1.5.12. The conversion of 2'-F-ara-ddP to 2'-F-ara-ddI.

biotransformation was also detected in the brain homogenate, although the rate of conversion was slow and incomplete. An *in vivo* study in mice showed that the concentration of 2'-F-ara-ddI following oral administration of 2'-F-ara-ddP was significantly higher in brain than that after 2'-F-ara-ddI, indicating that 2'-F-ara-ddP is a brain-targeted prodrug of 2'-F-ara-ddI.

Although many prodrugs to AZT and other ddNs have been proposed, these research efforts have not resulted in any product marketed yet. Further research to identify prodrugs with clear advantages over AZT and other nucleoside RT inhibitors is needed.

In an attempt to increase the brain uptake and the retention of nucleoside RT inhibitors in plasma, another class of pyrimidine nucleoside prodrugs developed earlier has now been applied to nucleoside RT inbibitors. In this regard, 5-halo-6-alkoxy (or azido)-5,6-dihydro analogs were therefore designed in our research program as novel anti-HIV agents and/or potential prodrugs to AZT, d4T and FLT by Rakesh Kumar.[313-315] The chemical structures of these 5,6-dihydro analogs are shown in Figures 1.5.13.-1.5.15.

No.	X	R	Configuration	No.	X	R	Configuration
1	Br	OCH_3	5R,6R	13	Cl	$OCH(CH_3)_2$	5R,6R
2	Br	OCH_3	5S,6S	14	Br	$O(CH_2)_7CH_3$	5R,6R
3	Cl	OCH_3	5R,6R	15	Cl	$O(CH_2)_7CH_3$	5R,6R
4	Cl	OCH_3	5S,6R	16	Br	$O(CH_2)_{15}CH_3$	5R,6R
5	I	OCH_3	5R,6R	17	Cl	$O(CH_2)_{15}CH_3$	5R,6R
6	I	OCH_3	5S,6S	18	Br	N_3	5R,6R
7	Br	OC_2H_5	5R,6R	19	Br	N_3	5S,6S
8	Br	OC_2H_5	5S,6S	20	Cl	N_3	5R,6R
9	Cl	OC_2H_5	5R,6R	21	Cl	N_3	5S,6S
10	Cl	OC_2H_5	5S,6S	22	Cl	N_3	5S,6R
11	Cl	OC_2H_5	5S,6R	23	Cl	N_3	5R,6S
12	Br	$OCH(CH_3)_2$	5R,6R				

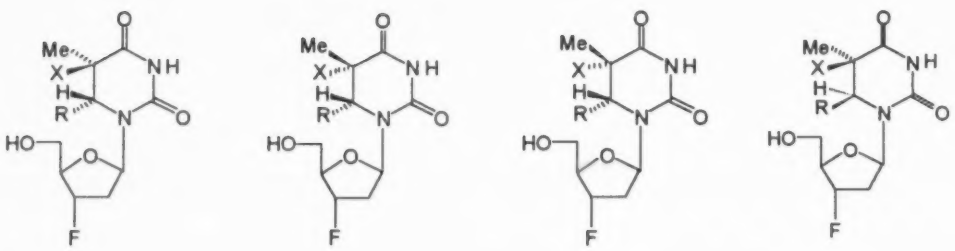


1,3,5,7,9,12-18,20 **2,6,8,10,19,21** **4,11,22** **23**

trans-(+)-(5R,6R) **trans-(-)-(5S,6S)** **cis-(+)-(5S,6R)** **cis-(-)-(5R,6S)**

Figure 1.5.13. The chemical structures of 5-halo-6-alkoxy (or azido)-5,6-dihydro analogs of AZT.

No.	X	R	Configuration	No.	X	R	Configuration
1	Br	OCH_3	5R,6R	6	I	OCH_3	5S,6S
2	Br	OCH_3	5S,6S	7	Br	N_3	5R,6R
3	Cl	OCH_3	5R,6R	8	Br	N_3	5S,6S
4	Cl	OCH_3	5S,6R	9	Br	N_3	5R,6S
5	I	OCH_3	5R,6R				

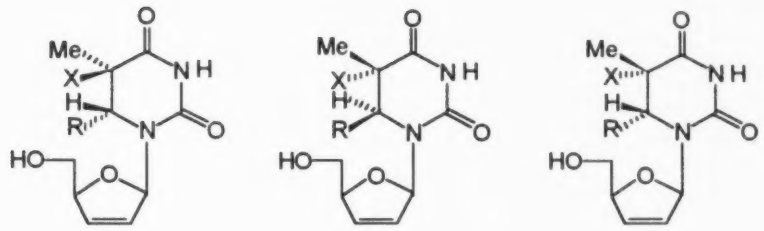


1,3,5,7 **2,6,8** **4** **9**

trans-(+)-(5R,6R) **trans-(-)-(5S,6S)** **cis-(+)-(5S,6R)** **cis-(+)-(5R,6S)**

Figure 1.5.14. The chemical structures of 5-halo-6-methoxy (or azido)-5,6-dihydro analogs of FLT.

No.	X	R	Configuration
1,2	Br	OCH_3	5R,6R; 5S,6S
3,4	Cl	OCH_3	5R,6R; 5S,6R
5,6	I	OCH_3	5R,6R; 5S,6S



1,3,5

trans-(+)-(5R,6R)

2,4

trans-(-)-(5S,6S)

6

cis-(+)-(5S,6R)

Figure 1.5.15. The chemical structures of 5-halo-6-methoxy-5,6-dihydro analogs of d4T.

2. Objectives

To address the general problems associated with nucleoside RT inhibitors (dose-limited bone marrow toxicity, insufficient brain uptake, fast clearance and reduced efficacy in macrophages), AZT has been the subject of extensive chemical modifications to produce more lipophilic ester prodrugs to enhance brain uptake, to sustain the release of AZT and to direct the delivery of AZT-5'-phosphate to macrophages. However, none of the modifications made so far has resulted in compounds with a clear-cut therapeutic advantage over AZT.

The objective of this project was to explore a new class of prodrugs of pyrimidine nucleoside RT inhibitors, 5-halo-6-alkoxy (or azido) -5,6-dihydro analogs, which putatively have the following advantages:

1. Increased brain uptake. It is known that HIV infection ultimately causes AIDS dementia, and that the degree of this condition is somewhat improved upon AZT therapy. However, the higher dose required for further improvement is limited by the toxicity. Secondly, other nucleoside RT inhibitors do not cross the BBB readily to provide a therapeutic drug concentration in brain. Therefore, effective brain delivery is considered to be important for better therapy.

2. Prolonged plasma half-life. Since AZT and other nucleoside RT inhibitors have short plasma half-lives, current therapy requires frequent dosing. Prolonged plasma half-lives will enhance their efficacy by providing longer elevated blood levels and by decreasing emphasis on dosing compliance.

3. Reduced toxicity. Bone marrow toxicity is a limiting factor for AIDS therapy using nucleoside RT inhibitors. A sustained release of AZT or other nucleoside RT inhibitors would prevent spikes in the plasma concentration, thereby reducing bone marrow toxicity since it is known that bone marrow toxicity is dose-related. Decreased

delivery to the marrow, relative to given blood levels, would also elevate the therapeutic index of these drugs.

To achieve the above objectives, a prodrug should have increased lipophilicity, slower clearance and undergo optimal conversion to the active drug. As part of the project, this thesis includes an evaluation of the novel prodrugs of AZT, FLT and d4T from the following aspects:

1. Stability. Physical stability is a basic requirement for a successful prodrug.

2. Determination of partition coefficients for these prodrugs as a measure of lipophilicity. It is known that AZT and some other ddNs pass through the cell membrane by passive diffusion. Increased lipophilicity (partition coefficient) usually favors passive diffusion across cell membranes and the BBB. It is expected that the 5,6-dihydro analogs will be more lipophilic than the parent nucleosides.

3. Phosphorolysis by thymidine phosphorylase. Phosphorolysis is a catabolic process that results in the breakdown of a nucleoside molecule by cleavage of the glycosidic bond. Stability toward phosphorolysis *in vitro* may be used as an indicator of *in vivo* stability .

4. Determination of the interaction of these prodrugs with an NBMPR-sensitive transport system which facilitates thymidine influx in human erythrocytes. It has been shown that a thymidine transport inhibitor increases the efficacy of AZT. The inhibition constant (K_i) for thymidine zero-trans influx in the NBMPR-sensitive nucleoside transport process represents the relative affinity of the test compound for this transport system.

5. Regeneration of the parent nucleoside from the prodrug *in vitro*. The usefulness of a prodrug depends largely on its regeneration to the active agent. The regeneration of the parent nucleoside from the prodrug by glutathione, whole mouse blood and the soluble enzyme fraction of mouse liver provides simple models for studying the mechanism of conversion of the prodrug to the parent drug.

6. Determination of AZT concentrations in blood after administration of the prodrug to mice. This experiment will clarify the blood concentration of AZT obtained from the prodrug, and whether the prodrug can provide a sustained release of AZT *in vivo*.

7. The biodistribution study will provide direct evidence for the potential of these prodrugs to enter the brain. Increased brain uptake is the primary objective of this research.

8. Distribution in brain subcellular fractions. This experiment will demonstrate if binding to any specific subcellular component is involved in the uptake of AZT, or its prodrugs, by brain tissue.

9. Distribution in regions of the brain will determine whether there is any regional preference for uptake of AZT, or its prodrugs, in brain.

3. Experimental

3.1. Materials.

3.1.1. Chemicals, solvents and equipment.

All chemicals used were reagent grade unless otherwise specified. 1-Octanol (99%, Aldrich Chemical Company, Inc.) was distilled before use. Glutathione (reduced, 98%) was purchased from the Aldrich Chemical Company, Inc. All solvents used for HPLC analyses were of HPLC grade and were purchased from Mallinckrodt and degassed prior to use. Thymidine was obtained from the Sigma Chemical Company. Protosol and Aquasol-2 were obtained from New England Nuclear and AZT was purchased from the Aldrich Chemical Company, Inc. A mechanical shaker (Dubnoff metabolic shaking incubator, Precision Scientific Co.) was used for partition coefficient determination. Eppendorf micropipettes were used for measurement of micro quantities. All centrifugations were performed using an Eppendorf 5412 microcentrifuge and Eppendorf microcentrifuge tubes unless otherwise specified. A PHM82 Standard pH Meter (Radiometer Copenhagen) was used for pH measurements. A HAAKE E3 water bath was used for controlled temperature experiments. A Vortex-Gene (Scientific Industries, Inc. Bohemia, N. Y. 11716, U. S. A.) was used for mixing small samples. Carbon-14 cocktail and combustion paper cups were purchased from R. J. Harvey Instrument Corporation, Hillsdale, N. J., U. S. A., and ScintiVerse™ II was purchased from the Fisher Scientific Company, Chemical Manufacturing Division, Fair Lawn, N. J., U. S. A. A Wittner (made in Germany) audible metronome was used for precise timing which was required for the transport study. A Spencer hemocytometer/Carsen microscope was used for erythrocyte counting.

3.1.2. Instruments.

Waters High Pressure Liquid Chromatography (HPLC) systems (model 501 pumps, model U6K injector, Hewlett Packard 1040A Photodiode-detection-system or Waters 486 variable wavelength absorbance detector) with either a HP 79994A workstation or a Waters system interface module were used for HPLC analysis. A C18 Radial-PAK cartridge (8 mm I.D., 10 cm length, 10 μm particle size) or a Whatman Partisil ODS-3 column (25 cm) was used. This type of HPLC system was also used for preparative purification of C-14 labeled compounds with the Whatman Partisil ODS-3 column (25 cm). A Beckman LS 9000 liquid scintillation counter was used for counting of ^{3}H and ^{14}C samples. A Biological Oxidizer OX300 (R. J. Harvey Instrument Corporation, Hillsdale, N. J., U. S. A.) was used for the quantitative combustion of tissue samples. An IEC B-20A Centrifuge (Damon/IEC Division) and a Beckman L7-55 Ultracentrifuge were used for the preparation of subcellular brain fractions.

3.1.3. Radioisotopes.

[Methyl-^{3}H]-thymidine (40-60 Ci/mmol, 1 mCi/mL) and ^{14}C-hexadecane (0.866 x 10^{6} dpm/mL on 1 July, 1988) were purchased from the Amersham Corporation, Arlington Hts, IL, U. S. A. [2-^{14}C]-AZT (56 mCi/mmol) was purchased from Moravek Biochemicals, Inc. (Brea, CA, U. S. A.). ^{14}C-Labeled 5-halo-6-methoxy (or ethoxy)-5,6-dihydro analogs of AZT were prepared from [2-^{14}C]-AZT.[303]

3.1.4. Animals.

Male and female Balb/C mice 20-25 g in weight and male $B_6D_2F_1$ mice weighing 25-30 g were purchased from the Health Sciences Small Animal Program, University of Alberta. Animals were handled in accordance with the guidelines of the Canadian Council on Animal Care and experimental protocols were approved by the University of Alberta Health Sciences Animal Welfare Committee.

3.1.5. Enzyme.

Thymidine phosphorylase from *E. coli* (1200 u/mL in 0.5 M potassium phosphate buffer containing 2 mM uracil, 0.02% sodium azide and bovine serum albumin) was purchased from the Sigma Chemical Company.

3.2. Methods.

3.2.1. The stability of 5-halo-6-methoxy-5,6-dihydro analogs of AZT upon incubation with cell culture medium MEMS/10%FBS.

The stability of 5-halo-6-methoxy-5,6-dihydro analogs of AZT in cell culture medium used for CEM cells was examined by incubation of the individual test compound with MEM/10%FBS at a concentration of 1 mM at 37°C for up to 48 h. At specified time intervals (0.5, 1.0, 3, 6, 12, 24 and 48 h), portions of 50 μL of the reaction mixture were removed and mixed with equal volume of ethanol (ice-cold). The mixture was subjected to centrifugation for 3 min at 12,800 rpm. An aliquot of 25 μL was analyzed by a HPLC method.

HPLC analysis was carried out on a reverse phase C_{18} cartridge (8 mm I.D., 10 cm length, 10 μm particle size) using methanol:water (40:60) at a flow rate of 1 mL/min with UV detection set at 230 nm.

3.2.2. Determination of partition coefficient.

The 1-octanol / water partition coefficients of AZT, FLT, d4T and their 5-halo-6-methoxy-5,6-dihydro analogs were measured using a literature method as described in the following paragraphs.[304]

3.2.2.1. Solvent preparation.

1-Octanol was refluxed for 4 h prior to distillation and the fraction having a boiling point of 196°C was collected. A mixture consisting of equal volumes of 1-octanol and water (HPLC grade) was gently stirred overnight. The water-saturated 1-octanol and 1-octanol-saturated water layers were then separated using a separatory funnel and each fraction was stored in a brown glass bottle at room temperature for subsequent use.

3.2.2.2. Preparation of the calibration curve for analysis by HPLC or UV spectrometry.

Standard solutions of the test compound were prepared in either 1-octanol-saturated water or in water-saturated 1-octanol, depending on which layer would be analyzed after equilibration. These standard samples were analyzed either by HPLC or by UV spectrometry depending on which method was used for the partitioned samples.

HPLC analyses were performed using a Partisil 5, ODS-3, 25 cm column with UV detection at 230 nm (for 5,6-dihydro analogs) or 265 nm (for parent drugs), using water:methanol (6:4 v/v; 1 mL/min) as eluting solvent. In the case that UV measurements were used, the wavelength of 230 nm for the 5,6-dihydro analogs and 265 nm for the parent drugs were chosen using a Shimadzu UV 160 spectrophotometer. Standard curves were prepared by plotting either HPLC peak area or UV absorbance vs. concentration of the standard solutions.

3.2.2.3. Determination of partition coefficients.

A known amount of the test compound was partitioned between equal volumes of 1-octanol-saturated water and water-saturated 1-octanol. The amount of the test compound was chosen so that the UV absorbance of the sample assayed after partitioning had an optimum value for either UV measurement or HPLC analysis. To ensure complete partitioning, the test tubes were shaken for 4.5 h at room temperature by a mechanical

shaker (Dubnoff metabolic shaking incubator, Precision Scientific Co.). The two phases were then separated. The concentration of test compound in the water layer was measured except for the 5-halo-6-methoxy-5,6-dihydro analogs of AZT, in which the test compound was measured in the 1-octanol layer rather than in water layer, because of their high lipophilicities. The concentration of test compound in the other phase was obtained by difference.

The partition coefficient (P value) was calculated from the following equation

$$P = C_{1\text{-octanol}}/C_{water}$$

where $C_{1\text{-octanol}}$ refers to the concentration of the test compound in the 1-octanol layer after partitioning, and C_{water} refers to its concentration in the water layer after partitioning.

3.2.3. Nucleoside transport studies.

The interaction between 5,6-dihydro analogs of AZT and the NBMPR-sensitive nucleoside transporter was examined in mouse erythrocytes by determination of inhibitory effects of test compounds on zero-trans influx of thymidine according to the literature method.[305,306] Zero-trans influx refers the movement of molecules of a compound from one side of the membrane to the other side where the concentration of the compound is to zero.

3.2.3.1. Preparation of mouse erythrocytes.

Male $B_6D_2F_1$ mice were asphyxiated with carbon dioxide in a 1000 mL beaker. The blood was collected by cardiac puncture in a 1.0 mL syringe containing 0.1 mL of 3.8% sodium citrate solution. The blood was used the same day.

The blood sample was transferred from the syringe to a 1.5 mL Eppendorf microcentrifuge tube and centrifuged for 1 min at 12,800 rpm. The supernatant was removed by pipette and the erythrocytes were then washed four times with cold HEPES buffered saline (HBS) which consisted of 140 mM NaCl, 18 mM HEPES and 1.8 mM $MgSO_4$ adjusted to pH 7.4 with 1N NaOH. The cells were then suspended in the same buffered saline at a hematocrit of 10%. This cell suspension was used for the transport study that was performed on the same day. The number of cells in the suspension was determined with a hemocytometer/microscope. The diluted cell suspension, prepared by mixing 10 μL of the erythrocyte suspension with 1.99 mL of HBS, was loaded on the chamber until the grid area was just full, but not overflowing. If the chamber was over-loaded, it was cleaned and reloaded. After the cells had settled, the total number of cells contained in 5 of the 25 smaller squares (the four corners and the center) were counted. The chamber was washed and the same procedure was repeated. The average value was calculated as follows and used for subsequent calculations.

cells/assay = (the number in 5 areas) x 5 x 10^4 x 200 ÷ 5

3.2.3.2. Thymidine influx time course.

The erythrocyte suspension (0.2 mL) was transferred to each 1.5 mL microcentrifuge tube. The thymidine influx time course was studied with [methyl-^{3}H]-thymidine (0.2 mL of 285 μM) which was prepared by mixing 142 μL of 10 mM thymidine, 60 μL of [methyl-^{3}H]-thymidine (specific activity 56 Ci/mmol, 1 mCi/mL) and 4.818 mL of HBS. The membrane transport of thymidine into erythrocytes was initiated by addition of the permeant to the erythrocytes. After specified time intervals (2, 4, 6, 8, 10, 20 and 30 s), the transport process was terminated by rapid addition of 0.4 mL NBMPR stopping solution (20 μM) followed by vortex (4 s) and centrifugation (1 min) at 12,800 rpm. The supernatant was aspirated and the resultant pellet was washed once with 1.0 mL of the same stopping solution. Perchloric acid (5%, 0.5 mL) was then

added to the resultant pellets to extract the intracellular [methyl-^{3}H]-thymidine, and the mixture was vortexed for 10 s. After at least 30 min at ice bath temperature, the mixture was subjected to centrifugation at 12,800 rpm for 3 min. Two aliquots (150 μL) of the supernatant were mixed with 15 mL of ScintiVerse II and counted using a liquid scintillation counter with a standard window setting for ^{3}H and a preset time of 10 min. Experiments were performed in duplicate samples.

Nonspecific absorption of thymidine to the erythrocytes (radioactivity trapped in the extracellular space of the pellet) was determined as the ***zero-time*** value and obtained by exposing the erythrocytes to the stopping solution (20 μM NBMPR, 0.4 mL) for 5 s prior to the addition of 0.2 mL of [methyl-^{3}H]-thymidine (285 μM). The subsequent procedures were the same as described above for the influx experiment. The resultant value was then subtracted from all of the measured values of thymidine influx to determine the transport rate.

Acquisition of useful results in this experiment is very dependent on good technique, including precise timing which was facilitated by an audible metronome.

3.2.3.3. Thymidine influx competition study.

Four standard [methyl-^{3}H]-thymidine solutions (0.5 mM, 1.0 mM, 2.5 mM and 5.0 mM) were prepared. [Methyl-^{3}H]-thymidine solutions at four concentrations (0.1 mM - 1.0 mM), alone and together with four concentrations (0.1 - 2.5 mM) of test compound were prepared by mixing appropriate amounts of the standard [methyl-^{3}H]-thymidine solutions with a stock solution of test compound as well as HBS, so called permeant solutions (20 solutions) as shown in Table 3.2.1. The thymidine influx competition experiment was performed at room temperature according to the literature method.[305,306] Nucleoside influx was initiated by the rapid addition of 200 μL of the individual permeant solution to 200 μL of erythrocyte suspension. After 3 s, transport was terminated by addition of NBMPR at a final concentration of 10 μM. Cells were pelleted by

Table 3.2.1. Permeant solutions prepared for the thymidine influx competition study.

	1.0 mM Thd	0.5 mM Thd	0.2 mM Thd	0.1 mM Thd
HBS	100 µL Thd-a[b]	100 µL Thd-b[c]	100 µL Thd-c[d]	100 µL Thd-d[e]
	400 µL HBS	400 µL HBS	400 µL HBS	400 µL HBS
2.5 mM T C[a]	50 µL Thd-a	50 µL Thd-b	50 µL Thd-c	50 µL Thd-d
	125 µL T C-1[f]	125 µL T C-1	125 µL T C-1	125 µL T C-1
	75 µL HBS	75 µL HBS	75 µL HBS	75 µL HBS
1.0 mM T C	50 µL Thd-a	50 µL Thd-b	50 µL Thd-c	50 µL Thd-d
	50 µL T C-1	50 µL T C-1	50 µL T C-1	50 µL T C-1
	150 µL HBS	150 µL HBS	150 µL HBS	150 µL HBS
0.5 mM T C	50 µL Thd-a	50 µL Thd-b	50 µL Thd-c	50 µL Thd-d
	125 µL T C-2[g]	125 µL T C-2	125 µL T C-2	125 µL T C-2
	75 µL HBS	75 µL HBS	75 µL HBS	75 µL HBS
0.1 mM T C	50 µL Thd-a	50 µL Thd-b	50 µL Thd-c	50 µL Thd-d
	25 µL T C-2	25 µL T C-2	25 µL T C-2	25 µL T C-2
	175 µL HBS	175 µL HBS	175 µL HBS	175 µL HBS

a. T C = Test compound. b. Thd-a = 5 mM [^{3}H]-Thd. c. Thd-b = 2.5 mM [^{3}H]-Thd.

d. Thd-c = 1.0 mM [^{3}H]-Thd. e. Thd-d = 0.5 mM [^{3}H]-Thd.

f. T C-1 = 5 mM Test compound. g. T C-2 = 1 mM Test compound.

centrifugation at 12,800 rpm for 1 min and washed once with 1 mL of the same NBMPR solution (20 µM). Cell pellets were extracted with 0.5 mL ice-chilled 5% perchloric acid. After at least 30 min cooling at ice temperature, the mixture was subjected to centrifugation and two aliquots (150 µL) of the supernatant were mixed with 15 mL of ScintiVerse™ II and counted. The ***zero-time*** value was obtained by reversing the order

in which NBMPR was added to the cells prior to the addition of the permeant solution. This value was subtracted from radioactivity values in all influx samples. The experiment was conducted in duplicate.

3.2.3.4. Liquid scintillation counting procedure.

To avoid color quenching, all samples were treated with 5% perchloric acid and placed in an ice-bath for 30 min prior to centrifugation for 3 min. Two aliquots (150 μL) were each mixed with 15 mL of ScintiVerse™ II. Perchloric acid (5%, 150 μL) in 15 mL of ScintiVerse™ II was used for the estimation of background counts. Each of the four [methyl-^{3}H]-thymidine standards (20 μL) was also mixed with 130 μL of 5% perchloric acid. All of the samples were counted at a window setting for standard ^{3}H with a preset time of 10 min.

3.2.3.5. Calculation of zero-trans influx.

The internal concentration of ^{3}H-thymidine in erythrocytes was calculated according to the following equation:

$$C = (G-Z)\ HF/S_p$$

Where C is given as nanomoles per 10^{10} cells.

G is the number of counts per minute of the sample.

Z is the number of counts per minute of the zero-time sample.

S_p is the specific activity in counts per minute (CPM) per microliter of loading medium per thymidine concentration given in mM.

F is a constant (conversion factor) which converts nmol ^{3}H-thymidine per pellet to nmol ^{3}H-thymidine per 10^{10} cells.

H is a constant (correction factor) which takes into account:

1. the dilution due to addition of HBS (10% hematocrit)

2. the aliquot of solution taken for counting (150 μL)

3. the cellular water fraction under the experimental conditions (70% water)

The velocity of uptake (nmol ^{3}H-thymidine /10^{10} cells/s), was obtained by dividing the internal concentration as calculated above, by the time elapsed (t). The Michaelis constant (K_m) and the maximum velocity (V_{max}) for the ***zero-time*** influx of thymidine were estimated by linear least squares regression analysis of s/v against s plots (s is the external thymidine concentration) according to the method of Hanes.[307] The absolute value of the intercept on the horizontal axis is K_m and the value of the intercept on the vertical axis is K_m/V_{max}.

The inhibition constant K_i was estimated by plotting 1/v against i (i is the concentration of the test compound) according to the method of Dixon[308] and the lines were fitted by the visual best fit and intersected at a point, for which $i = -K_i$.

3.2.4. *In vitro* phosphorolysis studies using *E. coli* thymidine phosphorylase.

Phosphorolysis studies were carried out by incubation of the individual test compound with *E. coli* thymidine phosphorylase according to a literature method.[309,310]

3.2.4.1. Preparation of Tris buffer solution (10 mM Tris-HCl, pH 7.3).

The Tris buffer solution was prepared by dissolving the Tris base (Trizma, 0.141 g) and $Na_2EDTA{\cdot}2H_2O$ (74.4 mg) in distilled water (90 mL). The pH was adjusted to 7.3 with 10 N HCl and the volume was adjusted to 100 mL using distilled water.

3.2.4.2. Preparation of potassium phosphate buffer (100 mM, pH 7.1).

Potassium dihydrogen phosphate (1.36 g) was dissolved in distilled water (90 mL) and the pH was adjusted to 7.1 with 10 N KOH. The solution volume was adjusted to 100 mL with distilled water.

3.2.4.3. Preparation of the diluted enzyme.

The enzyme was diluted the same day that the experiment was performed. A 5 μL aliquot of the stock enzyme solution (thymidine phosphorylase from *E. coli*, Sigma T-7006, 1200 u/mL in 0.5 M potassium phosphate buffer containing 2 mM uracil, 0.02% sodium azide and bovine serum albumin) was diluted with Tris buffer (3.9 mL). This solution was maintained at ice-bath temperature prior to use. One unit of the enzyme is defined as the amount of enzyme that cleaves 1 μmole of thymidine in 1 min at 37°C.

3.2.4.4. Preparation of test compound solutions.

A 5 mM solution of each test substrate in Tris buffer was prepared, and the solutions were stored at 4°C for subsequent use.

3.2.4.5. Thymidine phosphorolysis time course.

Thymidine (250 nmol) was incubated with 130 μL of the diluted enzyme solution (0.2 u of *E. coli* thymidine phosphorylase) at 37°C. The samples were taken at 5, 10, 20, 30, 60 and 360 min after the incubation and analyzed by a HPLC method. The HPLC analysis was performed using a Whatman partisil ODS-3 column (25 cm) with methanol:water (5:95) at 2 mL/min and UV detection at 270 nm. Retention times for thymine and thymidine were 14.0 and 20.2 min, respectively.

3.2.4.6. Stability of 5-halo-6-methoxy-5,6-dihydro analogs toward phosphorolysis.

An *in vitro* study of the phosphorolysis of Thd, AZT, d4T, 2'-deoxyuridine (DU), EDU and their 5,6-dihydro analogs, was then performed by incubating the individual test compound with the thymidine phosphorylase from *E. coli* at 37°C for 10 min.

The diluted enzyme solution (130 μL, 0.2 u of the thymidine phosphorylase) was transferred to a 1.5 mL microcentrifuge tube and 20 μL of potassium phosphate buffer was added with Vortex mixing. The closed tube was then warmed to 37°C (HAAKE E3

water bath) for about 2 min. The phosphorolysis reaction was initiated by adding the substrate solution (50 μL, 250 nmoles) to the prewarmed mixture. The contents of the closed tube were mixed well prior to a 10 min incubation at 37°C. The reaction was terminated by removing the tube from the water bath and adding ice-cold methanol (200 μL) with thorough Vortex mixing. The tube was placed in an ice bath for 10 min and then centrifuged at 12,800 rpm in an Eppendorf microcentrifuge for 3 min at 4°C. An aliquot of the clear supernatant was subjected to HPLC analysis or stored in a freezer for subsequent analysis. Each experiment was performed at least twice.

HPLC analyses were performed using a C_{18} Radial-PAK cartridge (8 mm I.D., 10 cm length, 10 μm particle size) with UV detection at 230 nm, using water:methanol concentration 7:3 as eluent at a flow rate of 2 mL/min.

Thymidine was used as a reference compound to confirm the phosphorolysis activity of the enzyme.

3.2.5. The stability of 5,6-dihydro analogs in phosphate buffer.

The stability of 5-halo-6-methoxy-5,6-dihydro analogs was investigated upon incubation of 5-halo-6-methoxy-5,6-dihydro analogs of AZT with potassium phosphate buffer (pH 7.0, 0.06M) at a concentration of 1.0 mM at 37°C. After 1, 3, 5, 10 and 24 h, portions of 20 μL were removed from each reaction mixture and subjected to HPLC analysis.

HPLC analysis was performed on a reverse phase C18 cartridge using methanol:water (40:60) at 1 mL/min. The UV detector was set at 230 nm since the 5,6-dihydro analogs have low absorbance at 265 nm.

3.2.6. *In vitro* regeneration of parent drug from 5-halo-6-methoxy-5,6-dihydro analogs.

The *in vitro* regeneration of parent drug from the corresponding 5-halo-6-methoxy-5,6-dihydro analogs was studied upon incubation of the test compounds with glutathione, mouse whole blood and the soluble enzyme fraction of mouse liver.

3.2.6.1. The regeneration of parent drug from 5-halo-6-methoxy-5,6-dihydro analogs by glutathione.

The chemical regeneration of parent drug from 5-halo-6-methoxy-5,6-dihydro analogs of AZT, d4T and FLT has been investigated by incubation of the individual test compound with glutathione (reduced) according to a literature method[231] with modifications as subsequently described.

Phosphate buffer (pH 7.0, 0.06 M) was prepared by dissolving potassium dihydrogen phosphate (0.8142 g) in distilled water (90 mL) and the pH was adjusted to 7.0 with 1.0 N KOH. The solution volume was then adjusted to 100 mL with distilled water. Glutathione solution was freshly prepared on the day of the experiment by dissolving glutathione in the phosphate buffer. The test substrate solution was also dissolved in phosphate buffer.

The test substrate solution was mixed with the glutathione solution using the molar ratios specified in the "Results and Discussion" Section. This mixture was then incubated at 37°C for various time periods up to 24 h. The resultant sample was analyzed by HPLC.

HPLC analyses were conducted using a C_{18} Radial-PAK Cartridge (8 mm I.D., 10 cm length, 10 μm particle size) with UV detection at both 230 nm and 265 nm using water:methanol 7:3 (v/v) as eluent at a flow rate of 2 mL/min.

The appearance of parent drug (AZT or FLT or d4T) at their known retention times, after the 5-halo-5-methoxy-5,6-dihydro analogs were incubated with glutathione,

indicated that regeneration of the 5,6-double bond had occurred. The % regeneration was determined using a standard curve prepared by plotting peak area vs. concentration.

3.2.6.2. Regeneration of parent drug from 5-halo-6-methoxy-5,6-dihydro analogs by mouse whole blood.

The regeneration of parent drug from 5-halo-6-methoxy-5,6-dihydro analogs was studied by incubation of the individual test compound with heparinized blood from a Balb/c mouse.

Blood was drawn into a heparinized syringe from mice by heart puncture after the Balb/c mice were asphyxiated with carbon dioxide. The blood (100 μL) was transferred to each of the 1.5 mL microcentrifuge tubes and mixed with 100 μL of stock solution of test compound (1 mg/mL) followed by incubation at 37°C for 10 min. To terminate the reaction, the microcentrifuge tubes were removed from the 37°C water bath, and 200 μL of ice-cold methanol was added and the mixture was vortexed. The resultant mixture was kept in ice for 10 min before centrifugation at 12,800 rpm for 3 min in a cold room (4°C). The supernatant (200 μL) thus obtained was dried under a stream of N_2 at 35°C. The residue was then re-dissolved in 100 μL of methanol and an aliquot of 40 μL was subjected to HPLC analysis.

The HPLC analysis was performed using a reverse phase column C_{18} cartridge (8 mm I.D., 10 cm length, 10 μm particle size) with methanol:water (3:7) at 2 mL/min as eluent solvent. The UV detector was set to monitor absorbance at 230 nm and 265 nm.

3.2.6.3. Regeneration of parent drug from 5-halo-6-methoxy-5,6-dihydro analogs by the soluble enzyme fraction of mouse liver.

Preparation of soluble enzyme fraction of liver.

Three Balb/c mice were decapitated, and the livers were removed and washed with ice-cold 0.1 M Tris buffer (pH 8.2). The livers were homogenized in 2 volumes (w/v) of

the same buffer. The homogenate was centrifuged at 49,000 x *g* for 2 h at 4°C. The supernatant was then used to study the regeneration of parent drug from the corresponding 5,6-dihydro analogs.

Each individual test compound (100 μL, stock solution 1 mg/mL) was incubated with the soluble enzyme fraction of mouse liver (50 μL) at 37°C for half an hour. The reaction mixture was removed and mixed with an equal volume of methanol and subjected to centrifugation at 12,800 rpm for 3 min. The supernatant (200 μL) was dried under a stream of N_2 and redissolved in 100 μL of methanol. An aliquot of 25 μL was analyzed by HPLC method as described in Section 3.2.6.2.

3.2.7. Regeneration of AZT *in vivo* after i.v. administration of 5-halo-6-methoxy-5,6-dihydro analogs of AZT.

The *in vivo* regeneration of AZT from its 5-halo-6-methoxy-5,6-dihydro analogs was studied in mice after i.v. injection of the individual test compound.

The test compound solution (3.6 μmol/0.1 mL) was prepared in 10% (v/v) DMSO/water. The solution was injected into male or female Balb/c mice via the tail vein. The mice were sacrificed at desired time periods by asphyxiation with carbon dioxide. For each time point, 3 mice were used. The blood was then drawn into a heparinized syringe by cardiac puncture.

Each of the blood samples was mixed with 10 μg of 1-(2'-fluoro-2'-deoxy-β-D-arabinofuranosyl)-5-iodouracil (FIAU) or 5-bromo-6-methoxy-5,6-dihydro-3'-fluoro-3'-deoxythymidine (FLTBrOMe) as internal standard and centrifuged at 12,800 rpm for 1 min, and the supernatant was taken for extraction by Sep-Pak™.[74] The Sep-Pak cartridge was first rinsed with 2 mL of methanol followed by 5 mL of water. The blood sample was loaded onto the conditioned cartridge, which was then washed by passing 5 mL of water through the device. Methanol (2 mL) was used to elute the nucleosides/nucleoside derivatives. The methanol extract was dried under a stream of N_2 at 35°C, the resultant

residue was re-dissolved in 50 μL of methanol and an aliquot of 20 μL was injected into the HPLC for analysis. HPLC analysis was performed using a C_{18} cartridge with a mobile phase of water:methanol (7:3 v/v) at a flow rate of 2 mL/min, with UV detection at 265 nm and 230 nm. Sensitivity for AZT and the 5,6-dihydro analogs was 0.1 μg/mL and 1 μ g/mL, respectively. The recoveries of AZT, FIAU and FLTBrOMe by Sep-Pak extraction are 88%, 84% and 71%, respectively.

The amount of AZT in blood was quantitated using a standard curve which is a plot of peak ratio (AZT/FIAU or AZT/FLTBrOMe) vs. amount of AZT.

3.2.8. Biodistribution studies.

Biodistribution of [2-^{14}C]-AZT and its 5-bromo-6-methoxy (or ethoxy)-5,6-dihydro analogs were investigated in Balb/c mice after i.v. injection.

3.2.8.1. Preparation of the samples.

The radiolabeled compounds, [2-^{14}C]-5-bromo-6-methoxy (or ethoxy)-5,6-dihydro analogs of AZT (56 mCi/mmol, purity > 98%), were synthesized[303] from [2-^{14}C]-AZT which was purchased from Moravek Biochemical Inc. Two diastereomers of [2-^{14}C]-5-bromo-6-methoxy-5,6-dihydro analogs of AZT were separated by reverse phase HPLC using a Whatman Partisil ODS-3 (25 cm length) column, 80:20 water:methanol at 1.5 mL/min as eluent, with the UV detector set at 230 nm. The purified individual diastereomers were used for biodistribution studies. [2-^{14}C]-AZT, [2-^{14}C]-trans-(+)-(5R,6R)-, [2-^{14}C]-trans-(-)-(5S,6S)-5-bromo-6-methoxy-5,6-dihydro and [2-^{14}C]-trans-(+)-(5R,6R)-5-bromo-6-ethoxy-5,6-dihydro analogs of AZT were dissolved in sufficient water to make 20 μCi/ mL solutions.

3.2.8.2. Administration and biological sample collection.

A solution of test compound (100 μL; 2 μCi) was injected intravenously into each mouse via the tail vein. At different time intervals up to 8 h post administration, the mice were sacrificed by asphyxiation in a 800 mL beaker with carbon dioxide over which a watch glass was placed to collect excreted urine. Three mice were used for each time point. The blood was drawn by heart puncture. Several tissues including heart, liver, spleen, lung, kidney, blood, bone, brain and bladder were collected and weighed (wet) in the combustion cups. The weight of each tissue sample was limited to less than 200 mg with the exception of blood (0.2 mL) and urine, where varying volumes were taken. The tissues were allowed to dry at room temperature.

3.2.8.3. Sample analysis

The biological tissues prepared by the above procedure were analyzed for ^{14}C by a combustion/liquid scintillation counting method, for which a Biological Oxidizer OX300 and a Beckman LS 9000 liquid scintillation counter were used. The catalyst zone temperature of the oxidizer was heated to 685°C and the combustion zone to 900°C during a 30 min period prior to use. The nitrogen and oxygen flows were adjusted to 350-400 mL/min and the time of combustion was set at 4 min for each sample. The $^{14}CO_2$ produced from the tissues by combustion was trapped in plastic scintillation vials containing Harvey carbon-14 cocktail and radioactivity was determined by liquid scintillation counting. The combustion efficiency was determined by combusting standard samples (^{14}C-hexadecane and/or ^{14}C-AZT) containing known amounts of ^{14}C and calculating the ratio of the CPM from a standard sample after combustion over the CPM of the same amount of standard sample without combustion. The counting efficiency was calculated as CPM/dpm of a known amount of ^{14}C-hexadecane. After achieving stable results with standard samples, tissue samples were then analyzed. All resultant samples

were counted with a window setting for standard ^{14}C and a preset time of 10 min, after dark adaptation to eliminate chemiluminescence and phosphorescence.

3.2.9. Distribution of [2-^{14}C]-AZT and its [2-^{14}C]-5-bromo-6-methoxy (or ethoxy)-5,6-dihydro analogs in subcellular fractions of mouse brain.

A subcellular distribution study in mouse brain after jugular vein injection of [2-^{14}C]-AZT and its [2-^{14}C]-5-bromo-6-alkoxy-5,6-dihydro analogs was performed according to the literature procedure[311] with minor modifications.

3.2.9.1. Preparation of subcellular fractions of brain.

The brains obtained from 5 mice were rinsed with a few drops of ice-cold 0.32 M sucrose solution and the cerebella were removed. All of the other portions of the brains were weighed and placed in sufficient ice-cold 0.32 M sucrose solution to make a final homogenate concentration of 20% (w/v). The brain-sucrose solution was homogenized in a homogenizing tube by twelve reciprocal strokes with a Teflon plunger. The homogenization process was interrupted for about 1 min after the first six strokes to allow cooling by putting the tube in an ice-bath before continuing with the next six strokes. The resultant brain homogenate was then subjected to centrifugations at 0-4°C (shown in Figure 3.2.9.1). The P_1 pellet which contains nuclei and cell debris was obtained by centrifugation of the crude homogenate at 1000 x *g* for 10 min followed by two washes with the same sucrose solution using the same centrifugation conditions. The washings and the supernatant (S_1) were combined. The mixture was centrifuged at 10,000 x *g* for 30 min and the pellet was washed once. The resultant pellet P_2 contains myelin fragments, synaptosomes and mitochondria. The corresponding supernatant (S_2) and washing were combined and subjected to ultracentrifugation at 100,000 x *g* for 1 h to yield a microsomal pellet (P_3) and the supernatant S_3. All the fractions (P_1, P_2, P_3 and S_3) were frozen at -70°C for further analysis.

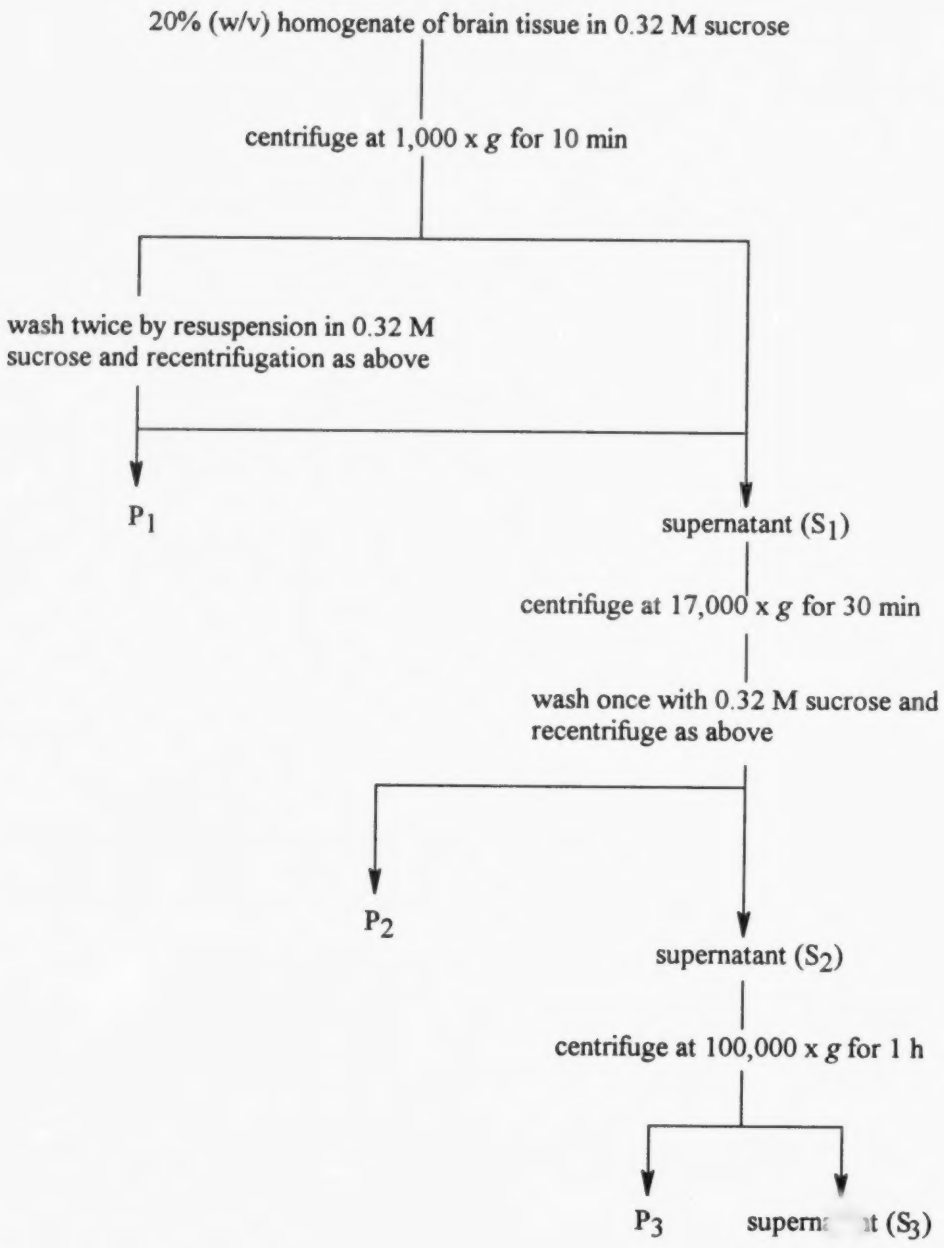

20% (w/v) homogenate of brain tissue in 0.32 M sucrose
centrifuge at 1,000 x g for 10 min
wash twice by resuspension in 0.32 M sucrose and recentrifugation as above
P1
supernatant (S1)
centrifuge at 17,000 x g for 30 min
wash once with 0.32 M sucrose and recentrifuge as above
P2
supernatant (S2)
centrifuge at 100,000 x g for 1 h
P3
supernatant (S3)

Figure 3.2.1. Flow diagram summarizing preparation of subcellular fractions of homogenized mouse brain.

3.2.9.2. Protein assay using the Lowry method.[312]

Preparation of reagents.

A. Bovine serum albumin (BSA). 1 mg/mL in distilled water was prepared as a stock solution. This solution can be prepared prior to use with storage at -70°C.

B. Membrane digestor. This solution was prepared by mixing 1 N NaOH and 1% Na deoxycholate (DOC) at a 1:1 ratio.

C Reagent A. Reagent A consists of the following constituents:
2% Na_2CO_3 : 1% $CuSO_4$: 2% NaKTartrate = 100 : 1 : 1 (v/v/v)

D. Folin Reagent (Folin-Phenol). A 1:1 mixture of commercial 2 N Folin and distilled water.

Solutions B, C and D must be prepared fresh just before use.

BSA is used to prepare the standard curve for analysis of protein contents in brain subcellular fractions. BSA (0-500 μL), or the sample to be analyzed, was added to the test tubes and the total volume was adjusted to 800 μL with distilled water. Membrane digestor (200 μL) was added to each of the tubes with thorough mixing. After 10 min, reagent A was added and mixed by vortex. After waiting for another 10 min, Folin reagent was added with vortex mixing. The absorbance at 660 nm was measured after at least 30 min and a blank sample (no BSA) was used as the reference. All BSA standards and samples were analyzed in duplicate.

The absorbance at 660 nm was plotted against the amount of BSA in the standard solution. The best fit line through the data points was calculated by linear regression. The amount of protein in the unknown sample was determined by interpolation from the standard curve. To get optimum absorbance, P_1, P_2 and P_3 had to be diluted by a factor of 5 with distilled water.

3.2.9.3. Radioactivity determination.

The pellet fractions P_1, P_2 and P_3 were solubilized with basic solubilizer (Protosol™) at a sample : Protosol ratio of 1:1, at 55°C for about 30 min, followed by addition of 0.1 mL of glacial acetic acid to neutralize the samples and a few drops of freshly prepared 4% stannous chloride in 0.1 N HCl. The samples were then mixed with 15 mL of Aquasol-2™ scintillation fluor. Fraction S_3 (2 mL) was directly mixed with 15 mL of Aquasol-2. The samples were counted in a liquid scintillation counter at a window setting for ^{14}C, with a preset time of 10 min. The counted samples were mixed with 10 µL of ^{14}C standard, i.e. ^{14}C-hexadecane (0.866×10^6 dpm/mL on 1 July, 1988) and recounted. The counting efficiency thus obtained was used to correct the counting results from the samples.

3.2.9.4. Sample preparation and administration.

[2-^{14}C]-AZT, [2-^{14}C]-trans-(+)-(5R,6R)-5-bromo-6-methoxy-5,6-dihydro or [2-^{14}C]-trans-(+)-(5R,6R)-5-bromo-6-ethoxy-5,6-dihydro analogs of AZT were each dissolved in sufficient water to make a 2 µCi/0.1 mL solution for use. The mouse was anesthetized with methoxyflurane by inhalation and monitored closely using the toe pinch reflex. Once the effective and painfree anaethesia was achieved, an incision of approximately 3/8" was made and the pectoral muscle and jugular vein were exposed. The test compound was injected via the jugular vein (2 µCi in 0.1 mL to each mouse). Ten minutes after the injection, the mice were sacrificed, and the brains were removed and chilled in an ice-cold petri dish. All subsequent steps were carried out as described in Section 3.2.9.1. The resultant P_1, P_2, P_3 and S_3 fractions were subjected to protein analysis and radioactivity determination.

The result for each individual fraction is expressed as total radioactivity, dpm/mg of protein as well as % radioactivity/%protein.

3.2.10. Regional distribution of [2-^{14}C]-AZT and its [2-^{14}C]-5-bromo-6-methoxy (or ethoxy)-5,6-dihydro analogs of in mouse brain.

A study of the regional distribution of [2-^{14}C]-AZT, [2-^{14}C]-trans-(+)-(5R,6R)-5-bromo-6-methoxy-5,6-dihydro and [2-^{14}C]-trans-(+)-(5R,6R)-5-bromo-6-ethoxy-5,6-dihydro analogs of AZT in brain was conducted in male Balb/c mice after jugular vein injection of the individual test compound.

The [2-^{14}C]-AZT, [2-^{14}C]-trans-(+)-(5R,6R)-5-bromo-6-methoxy-5,6-dihydro or [2-^{14}C]-trans-(+)-(5R,6R)-5-bromo-6-ethoxy-5,6-dihydro analog of AZT was dissolved in sufficient distilled water to make a solution of 20 μCi/mL. The mouse was anesthetized with methoxyflurane using inhalation and monitored closely by the toe pinch reflex. Once effective and painfree anaethesia was achieved, an incision of approximately 3/8" was made and the pectoral muscle and jugular vein were exposed. [2-^{14}C]-AZT or its 5-bromo-6-methoxy (or ethoxy)-5,6-dihydro analogs (0.1 mL) was injected via the jugular vein. Ten minutes after the injection, the mice were sacrificed, and the brains were removed and maintained in saline at ice bath temperature. The brain was subsequently dissected into portions which include cerebellum, pons and medulla, hypothalamus, hippocampus, striatum, cortex and the rest of brain. The samples were pooled from four (AZT) or two (5-bromo-6-alkoxy-5,6-dihydro analogs) mice after administration of the compound and weighed wet. The parts were dried at room temperature overnight and subjected to standard combustion/liquid scintillation counting method as described in Section 3.2.8.3. The results were expressed as CPM/mg for the specific samples.

4. Results and Discussion

A number of 5-halo-6-alkoxy (or azido)-5,6-dihydro analogs of AZT, FLT and d4T have been designed as new anti-HIV agents and as potential prodrugs to AZT, FLT and d4T for brain targeting.[313-315] The structures have been shown in Figures 1.5.13.-1.5.15. (pages 52-54). Four diastereomers are possible due to the presence of chiral centers at the C^5 and C^6 positions of the dihydrouracil ring. The configurations at C^5 and C^6 were assigned by comparison to literature NMR data and the acquisition of an X-ray crystal structure for (+)-(5R,6R)-5-chloro-6-methoxy-5,6-dihydro-1-(2',3'-didehydro-2',3'-dideoxy-β-D-glycero-2'-enopentofuranosyl)thymine.[316] The major diastereomers obtained in the 5-iodo-6-alkoxy and 5-bromo-6-alkoxy (or azido) reactions possessed the trans-(+)-(5R,6R) and trans-(-)-(5S,6S) configurations. In contrast, the cis-(+)-(5S,6R) and trans-(+)-(5R,6R) diastereomers were obtained in the 5-chloro-6-alkoxy (or azido) syntheses.

As prodrugs, the differences between these novel 5,6-dihydro analogs and the ester prodrugs previously reported in the literature are that these 5-halo-6-alkoxy (or azido)-5,6-dihydro analogs have a) 5'-OH free, and b) modifications across the C^5, C^6 double bond.

4.1. *In vitro* cytotoxicities and anti-HIV activity of 5-halo-6-alkoxy (or azido)-5,6-dihydro analogs of AZT, FLT and d4T in HIV-1-infected CEM cells.

The *in vitro* anti-HIV activities and cytotoxicities of 5-halo-6-alkoxy (or azido)-5,6-dihydro analogs of AZT, FLT and d4T were evaluated by the U.S. National Cancer Institute Antiviral Evaluations Branch according to a literature method.[317] The ability of the test compound to protect HIV-1-infected T4 lymphocytes (CEM cells) from cell death

was determined. Small amounts of HIV are added to cells, and a complete cycle of virus replication is necessary to obtain the required cell killing. Agents that interact with virions, cells, or virus gene products to interfere with viral activities will protect cells from cytolysis. The EC_{50} value is the drug concentration which produces a 50% survival of HIV-infected cells relative to uninfected untreated controls and the IC_{50} value is the drug concentration which results in a 50% survival of uninfected untreated control CEM cells. The results are shown in Appendix I. The 5-iodo and 5-bromo-6-methoxy analogs exhibit EC_{50} values lower than their corresponding 5-chloro analogs with the EC_{50} values of 5-iodo-6-methoxy analogs generally similar to those of parent drugs. The results indicate that the relative *in vitro* anti-HIV activity of 5-halo-6-methoxy analogs is in the order of -I > -Br >Cl and the 5-iodo-6-methoxy analogs are approximately equipotent to their parent compounds. Among the 5-bromo-6-alkoxy analogs of AZT, the EC_{50} values generally increased when the size of the substituent at C^6 was increased or the alkoxy group becomes branched. However, the IC_{50} values remained about the same. In the case of the 5-chloro-6-alkoxy analogs of AZT, the compounds become inactive when the alkoxy groups at C^6 were bigger than ethoxy.[313-315] The results suggest that substituents at C^5 and C^6 are factors affecting the activity of the 5,6-dihydro analogs. The anti-HIV activities and cytotoxicities of these 5,6-disubstituted-5,6-dihydro analogs of AZT, FLT and d4T may reflect the activity of the individual compounds and/or may be related to the conversion of the 5,6-dihydro analogs to their parent compounds, since 5,6-dihydrouracils are known to convert to their parent compounds under various conditions.[231-233,318-323] The similarities between the activities of the parent drugs and their 5-iodo-6-methoxy-5,6-dihydro analogs may in fact be an indicator of fast regeneration of the parent drug. The decreased activities or inactivity observed for other 5-halo-6-alkoxy-5,6-dihydro analogs are likely due to slower regeneration or no regeneration of the parent drug. The 5-halo-6-methoxy-5,6-dihydro analogs of AZT were therefore selected for study of stability in the cell culture medium used for growth of CEM cells.

4.2. The stability of 5-halo-6-methoxy-5,6-dihydro analogs of AZT in MEMS/ 10%FBS.

Trans-(+)-(5R,6R)-5-chloro (bromo or iodo)-6-methoxy-5,6-dihydro analogs of AZT were incubated in the cell culture medium used for CEM cells, i.e., MEMS/10%FBS at a concentration of 1 mM at 37°C. After desired time periods, the samples were analyzed by HPLC. It was found that the trans-(+)-(5R,6R)-5-bromo (or iodo)-6-methoxy-5,6-dihydro analogs of AZT gradually converted to AZT (Figure 4.2.1.), while the trans-(+)-(5R,6R)-5-chloro-6-methoxy-5,6-dihydro analog is stable, with no AZT formed after the 5-chloro-6-methoxy-5,6-dihydro analog was incubated in the cell culture medium for 48 h. These results suggest that 5-bromo (or iodo)-6-methoxy-5,6-dihydro analogs of AZT may have been partially converted to AZT under the conditions of determining the EC_{50} and IC_{50}. It therefore seems rational to conclude that the EC_{50} and IC_{50} values obtained do not reflect the activity of the 5-bromo (or iodo)-6-methoxy-5,6-dihydro analogs, since they are the results obtained from mixtures of the 5,6-dihydro analogs and AZT. However, the conversion from 5-chloro-6-methoxy-5,6-dihydro analog of AZT under the same conditions may be negligible, so the EC_{50} and IC_{50} values determined for the chloro analog are the results for this compound. The 5-chloro-6-methoxy-5,6-dihydro analog demonstrated increased EC_{50} and IC_{50} values relative to 5-bromo (or iodo)-6-methoxy-5,6-dihydro analogs, suggesting that this 5,6-dihydro analog is less potent than AZT. Thus the EC_{50} and IC_{50} observed for 5-iodo (bromo)-6-alkoxy-5,6-dihydro analogs of AZT, FLT and d4T may reflect the conversion from the dihydro analogs to their corresponding parent compounds.

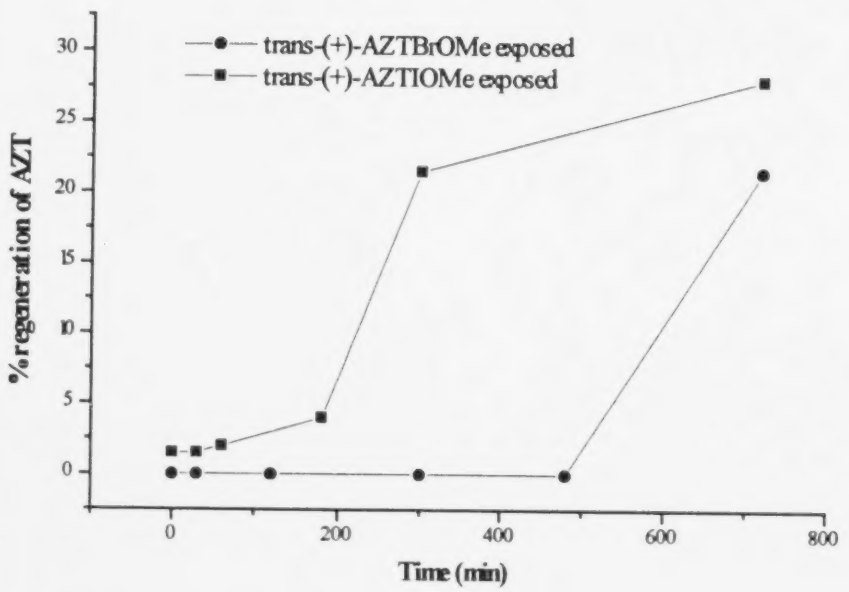


Figure 4.2.1. The regeneration of AZT from its 5-bromo (or iodo)-6-methoxy-5,6-dihydro analogs in MEMS/10%FBS at a concentration of 1 mM at 37°C.

4.3. Partition coefficients.

Strategies for drug delivery through the BBB involve the production of liposomes, drug lipidization and the development of lipid-soluble prodrugs, since the ability of a compound to traverse the cell membrane and the BBB is known to be at least partly dependent on the lipophilicity of the compound. However, liposomes have been generally ineffective because of their large sizes.[324] Our primary objective was to make prodrugs which are more lipophilic and therefore brain-targeted by virtue of their ability to diffuse readily across the BBB. The partition coefficient is a measure of lipophilicity and usually is considered to be an indicator of the ability of a compound to cross the cell membrane as well as the BBB into brain.[324,325]

Table 4.3.1. Partition coefficients (P) of thymidine, AZT and 5-halo-6-methoxy-5,6-dihydro analogs of AZT. Partition coefficients were determined in 1-octanol/water system at 37°C. $P = C_{1\text{-octanol}} / C_{water}$, where $C_{1\text{-octanol}}$ refers to the concentration of the test compound in the 1-octanol layer, and C_{water} refers to its concentration in the water layer after partitioning. The data shown are means ± SD, n = 5.

Compound	P value[a]
Thymidine	0.06[173]
Azidothymidine (AZT)	1.29 ± 0.12
trans-(+)-(5R,6R)-AZTClOMe	7.61± 2.81
cis-(+)-(5S,6R)-AZTClOMe	3.30 ± 1.10
trans-(+)-(5R,6R)-AZTBrOMe	13.21 ± 1.80
trans-(-)-(5S,6S)-AZTBrOMe	16.70 ± 5.23
trans-(+)-(5R,6R)-AZTIOMe	10.63 ± 1.41
trans-(-)-(5S,6S)-AZTIOMe	18.81 ± 1.13

The partition coefficients for AZT, FLT, d4T and their 5-halo-6-methoxy-5,6-dihydro analogs were determined in a 1-octanol/water system. The partition coefficient (P) is defined as $P = C_{1\text{-octanol}}/C_{water}$, at room temperature. The results, along with the reported partition coefficient for thymidine, are shown in Tables 4.3.1.- 4.3.3.

Compared with thymidine (P = 0.06)[173], AZT (P = 1.29), FLT (P = 0.5) and d4T (P = 0.12) are more lipophilic. Among them, AZT is the most lipophilic compound due to the $-N_3$ at the 3'- position of the sugar moiety. This is consistent with the fact that the azido substituent possesses very different physicochemical properties compared to the hydroxy group, especially steric size (molar refractivity value) and lipophilic (π-value) effects, which can be demonstrated from the results presented in Table 4.3.4.[326]

Table 4.3.2. Partition coefficients (P) of FLT and selected 5-halo-6-methoxy-5,6-dihydro analogs. Partition coefficients were determined in 1-octanol/water system at 37°C. $P = C_{1\text{-octanol}}/ C_{water}$, where $C_{1\text{-octanol}}$ refers to the concentration of the test compound in the 1-octanol layer, and C_{water} refers to its concentration in the water layer after partitioning. The data shown are means ± SD, n = 5.

Compound	P value
3'-Fluoro-3'-deoxythymidine (FLT)	0.50 ± 0.02
Trans-(+)-(5R,6R)-FLTClOCH$_3$	5.15 ± 0.28
Cis-(+)-(5S,6R)-FLTClOCH$_3$	1.50 ± 0.03
Trans-(+)-(5R,6R)-FLTBrOCH$_3$	4.71 ± 0.19
Trans-(-)-(5S,6S)-FLTBrOCH$_3$	3.44 ± 0.05
Trans-(+)-(5R,6R)-FLTIOCH$_3$	2.81 ± 0.09
Trans-(-)-(5S,6S)-FLTIOCH$_3$	4.00 ± 0.21

Table 4.3.3. Partition coefficients of d4T and selected 5-halo-6-methoxy-5,6-dihydro analogs. Partition coefficients were determined in 1-octanol/water system at 37°C. $P = C_{1\text{-octanol}} / C_{water}$, where $C_{1\text{-octanol}}$ refers to the concentration of the test compound in the 1-octanol layer, and C_{water} refers to its concentration in the water layer after partitioning. The data shown are means ± SD, n = 5.

Compound	P value
2',3'-Didehydro-2',3'-dideoxythymidine (d4T)	0.12 ± 0.02
Trans-(+)-(5R,6R)-d4TClOCH$_3$	2.60 ± 0.54
Cis-(+)-(5S,6R)-d4TClOCH$_3$	1.00 ± 0.14
Trans-(+)-(5R,6R)-d4TBrOCH$_3$	3.39 ± 0.29
Trans-(-)-(5S,6S)-d4TBrOCH$_3$	1.45 ± 0.12
Trans-(+)-(5R,6R)-d4TIOCH$_3$	2.60 ± 0.25
Trans-(-)-(5S,6S)-d4TIOCH3	1.25 ± 0.15

Table 4.3.4. Impact of -N_3 and -OH on molar refractivity and π values of a benzene ring.[327]

Substituent	Steric size effect (Molar refractivity value)	Lipophilic effect (π-value)
-N_3	10.2	+0.46
-OH	2.85	-0.67

The constant π was defined as $\pi_X = \log P_X - \log P_H$ by Fujita *et al*[327] and is used to correlate biological activity with chemical conposition. P_H is the partition coefficient of

a parent compound and P_X is that of a derivative of the parent compound with X as a substituent. A negative π value implies that the compound in question prefers the aqueous layer relative to the parent compound, and a positive π value indicates that the organic layer is preferred. By applying the same calculation to AZT and thymidine for the constant π (-N_3 vs. -OH), we found that π is equal to 1.30, which is close to the value of 1.13 attributed to their effects on a benzene ring.[327] The π value calculated for -F from FLT vs. thymidine is also determined as 0.90. To determine the π values of 5-halo and 6-methoxy, additional standard compounds (6-methoxy-5,6-dihydro and 5-halo-5,6-dihydro analogs) are needed. Molecular π values are therefore calculated relative to corresponding parent nucleoside analogs and are presented in Tables 4.3.5.-4.3.7. Trans-addition products generated similar π values (for AZT series, from 0.77 to 1.16; for FLT series, from 0.75 to 0.97 and for d4T series, from 1.08 to 1.45) while the cis-addition products have somewhat smaller effects.

Table 4.3.5. Impact of 5,6-disubstitutions on lipophilicity of AZT.

$\pi = \log P_X - \log P_H$, where P_X is the partition coefficient of the 5,6-dihydro analog in question and P_H is the partition coefficient of AZT.

Substituent	π value
trans-(+)-(5R,6R)-5-chloro-6-methoxy	+0.77
cis-(+)-(5S,6R)-5-chloro-6-methoxy	+0.41
trans-(+)-(5R,6R)-5-bromo-6-methoxy	+1.01
trans-(-)-(5S,6S)-5-bromo-6-methoxy	+1.11
trans-(+)-(5R,6R)-5-iodo-6-methoxy	+0.92
trans-(-)-(5S,6S)-5-iodo-6-methoxy	+1.16

Table 4.3.6. Impact of 5,6-disubstitutions on lipophilicity of FLT.

$\pi = \log P_X - \log P_H$, where P_X is the partition coefficient of the 5,6-dihydro analog in question and P_H is the partition coefficient of FLT.

Substituent	π value
trans-(+)-(5R,6R)-5-chloro-6-methoxy	+1.01
cis-(+)-(5S,6R)-5-chloro-6-methoxy	+0.48
trans-(+)-(5R,6R)-5-bromo-6-methoxy	+0.97
trans-(-)-(5S,6S)-5-bromo-6-methoxy	+0.84
trans-(+)-(5R,6R)-5-iodo-6-methoxy	+0.75
trans-(-)-(5S,6S)-5-iodo-6-methoxy	+0.90

Table 4.3.7. Impact of 5,6-disubstitutions on lipophilicity of d4T.

$\pi = \log P_X - \log P_H$, where P_X is the partition coefficient of the 5,6-dihydro analog in question and P_H is the partition coefficient of d4T.

Substituent	π value
trans-(+)-(5R,6R)-5-chloro-6-methoxy	+1.34
cis-(+)-(5S,6R)-5-chloro-6-methoxy	+0.92
trans-(+)-(5R,6R)-5-bromo-6-methoxy	+1.45
trans-(-)-(5S,6S)-5-bromo-6-methoxy	+1.08
trans-(+)-(5R,6R)-5-iodo-6-methoxy	+1.34
trans-(-)-(5S,6S)-5-iodo-6-methoxy	+1.02

In 1954, Collander[328] demonstrated that the rate at which many organic compounds passed through the cellular material of Nittela cells was proportional to their log P ($P \leq 8.5$). This finding was confirmed by Milorrow and Williams in 1968.[329] However, Hansch and Fujita argued that the linear relationship could only be expected

when P is within a limited range.[327] A molecule partitions between many "aqueous" phases and "organic" phases in going through the wall-membrane section before it reaches the site of action. A compound with excessive lipid solubility will no longer circulate in the bloodstream, but rather bind to the first lipid membrane it encounters. Therefore, it has been known for a long time that as one increases the partition coefficient of a biologically active compound, biological activity often rises, but after a certain point it falls off and eventually reaches zero.[327] Thus there is often an optimum partition coefficient for a biologically active class of compounds,[330] as shown in Figure 4.3.1. P_a is defined as biological activity and P_o is the optimal partition coefficient to achieve maximum biological effect.

It is known that the permeation of nucleosides through the BBB may be facilitated by increasing their lipophilicity, particularly for compounds with a molecular weight below 400.[331,355] The molecular weights of 5-halo-6-methoxy-5,6-dihydro analogs of AZT, FLT and d4T are in the range of 291 to 425 as shown in Table 4.3.8.

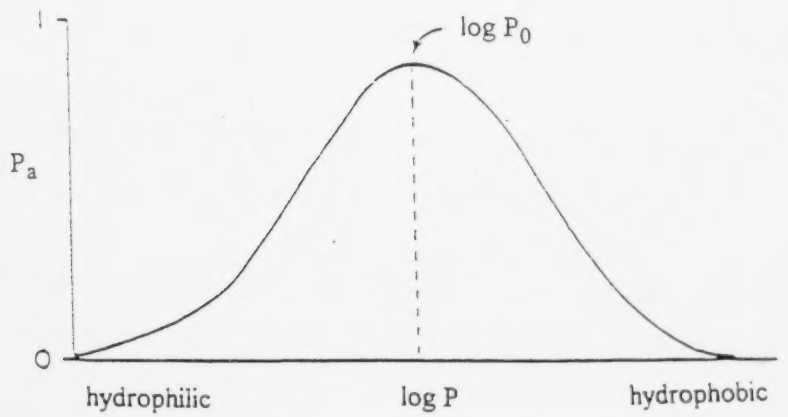


Figure 4.3.1. Relationship between biological activity (P_a) and lipophilicity (log P).

Table 4.3.8. Molecular weights of 5-halo-6-methoxy-5,6-dihydro analogs of AZT, FLT and d4T.

Substituent	Molecular Weight		
	AZT	FLT	d4T
5-Chloro-6-methoxy-5,6-dihydro	334	311	291
5-Bromo-6-methoxy-5,6-dihydro	378	355	335
5-Iodo-6-methoxy-5,6-dihydro	425	402	382

In this study, it has been found that all of the 5-halo-6-methoxy-5,6-dihydro analogs were more lipophilic than their corresponding 5,6-unsaturated parent compounds, i.e. AZT, FLT and d4T (Tables 4.3.1-4.3.3). The P values for the 5-halo-6-methoxy-5,6-dihydro analogs of AZT were 2.5 to 15 times greater than that of AZT. The P values of 5-halo-6-methoxy-5,6-dihydro analogs of FLT were 3 to 10-fold higher than that of FLT, and the 5-halo-6-methoxy-5,6-dihydro analogs of d4T were 8 to 28 times more lipophilic than d4T. The two diastereomers of each analog also possess different lipophilicities. Among the 5,6-dihydro analogs, cis-(+)-(5S,6R)-5-chloro-6-methoxy-5,6-dihydro analog has the lowest partition coefficient relative to all other corresponding trans-5,6-dihydro analogs. This is likely due to the larger dipole-dipole effect across the C^5 and C^6 chemical bond as the result of the cis- addition compared to other trans- analogs. These results indicate that the configurations of the C^5 and C^6 chiral centers in the two diastereomers are determinants of lipophilicity. These results are similar to a previous report on 5-halo-5-ethyl-6-methoxy-5,6-dihydro analogs of 5-ethyl-2'-deoxyuridine (EDU), which were shown to be more lipophilic (5- to 35-fold) than their parent drug, EDU.[232]

The relative lipophilicities of these compounds can also be observed from their retention times on reverse-phase HPLC chromatography. The more polar the compound, the smaller the retention time. The retention times of AZT and its 5-halo-6-methoxy-5,6-dihydro analogs are summarized in Table 4.3.9. Balzarini *et al*[332] reported that there was a correlation (correlation coefficient > 0.970) of P with t_R (retention time on HPLC) values for 36 anti-HIV nucleoside analogs that they investigated. Their results suggest that there are close linear correlations between the log P and t_R values of pyrimidine 2',3'-dideoxynucleosides, purine 2',3'-dideoxynucleosides, and 5-halogeno-substituted 3'-fluoro-2',3'-dideoxynucleosides. Correlation of log P with t_R for AZT and its 5-halo-6-methoxy-5,6-dihydro analogs also shows a linear relationship (Figure 4.3.2.) that is described by the following equation:

$$\log P = 0.061\ t_R - 0.2215 \quad \cdots\cdots\cdots\cdots \text{Equation 4.3.1.}$$

The correlation coefficient (R^2) for this relationship is 0.90. The linear relationship derived from 5-halo-6-methoxy-5,6-dihydro analogs of AZT may provide a useful tool to predict the partition coefficients of other 5-halo-6-alkoxy-5,6-dihydro analogs from their retention times under the same HPLC conditions. Although the P values for other 5-halo-6-alkoxy (other than methoxy)-5,6-dihydro analogs, were not determined, one can assume that elongation of the C^6 alkoxy chain increases the P-values.

According to Hansch and Fujita[327], there should be an optimum log P (log P_0) for achieving the best biological activity. It has been shown previously[355] that log P's between 0.9 and 2.5 are optimal for brain extractability of certain radiopharmaceuticals. However, without further studies employing additional compounds, this optimum log P_0 cannot be defined for the nucleosides used in the current study.

Table 4.3.9. Retention times of AZT and its 5-halo-6-methoxy-5,6-dihydro analogs on reverse phase HPLC. Column: Partisil 5, ODS-3, 25 cm; Mobile phase: methanol: water (60:40, v/v); flow rate: 1 mL/min; UV detection at 230 nm with the exception of 265 nm for AZT.

Compound	Retention time (min)
Azidothymidine (AZT)	7.5
cis-(+)-(5S,6R)-AZTClOMe	10.0
trans-(+)-(5R,6R)-AZTClOMe	21.0
trans-(+)-(5R,6R)-AZTBrOMe	20.5
trans-(-)-(5S,6S)-AZTBrOMe	24.0
trans-(+)-(5R,6R)-AZTIOMe	18.7
trans-(-)-(5S,6S)-AZTIOMe	24.0

It has been reported that AZT crosses the cell membrane of human lymphocytes by a nonfacilitated diffusion process (passive diffusion) and its uptake is insensitive to inhibitors of nucleoside transport.[173] Its unusual ability to cross the cell membrane is thought to be related to its partition coefficient, which is higher than that of other nucleosides.[173] Based on the previous findings that increased lipophilicity usually favors passive diffusion across cell membranes, it is postulated that the enhanced lipophilicity observed for the 5,6-dihydro analogs will increase the cellular uptake significantly. Other studies have demonstrated that prodrugs with increased lipophilicities possessed enhanced cellular uptake.[206,291] DP-AZT with a partition coefficient of 4.8 resulted in a 50% increase in cellular uptake compared with AZT.[291] Most 5'-ester prodrugs with higher partition coefficients (7.2-17.9) reported by Aggarwal *et al*[206] demonstrated increased cellular uptake (up to four-fold higher than AZT) and 5'-amino acid analogs of AZT with

decreased partition coefficients showed reduced ability to cross cell membranes. These results suggest that these 5'-amino acid analogs of AZT may not be transported by the active transport system used for amino acids and that the partition coefficient plays an important role in the diffusion of these AZT analogs into cells.

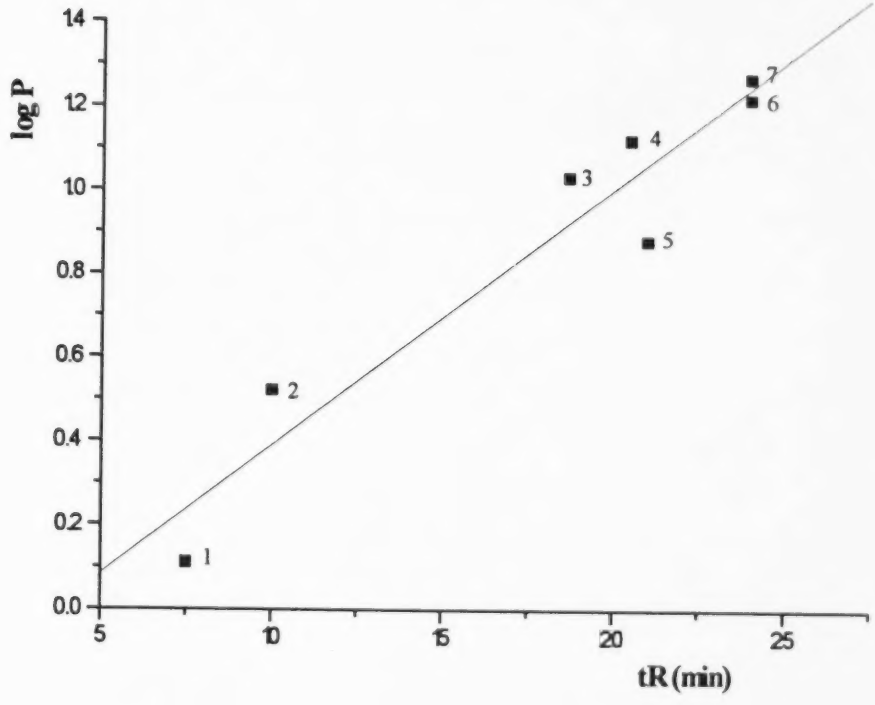


Figure 4.3.2. The correlation between lipophilicity (log P) and retention time (t_R) for reverse phase HPLC (Column: Partisil 5, ODS-3, 25 cm; Mobile phase: methanol: water (60:40, v/v); flow rate: 1 mL/min; UV detection at 230 nm with the exception of 265 nm for AZT.) 1 = AZT, 2 = cis-(+)-(5S,6R)-AZTClOMe, 3 = trans-(+)-(5R,6R)-AZTIOMe, 4 = trans-(+)-(5R,6R)-AZTBrOMe, 5 = trans-(+)-(5R,6R)-AZTClOMe, 6 = trans-(-)-(5S,6S)-AZTBrOMe, 7 = trans-(-)-(5S,6S)-AZTIOMe.

4.4. Interaction of 5-halo-6-methoxy-5,6-dihydro analogs of AZT with the NBMPR-sensitive nucleoside transport system in mouse erythrocytes.

Many nucleosides traverse cell membranes by facilitated mechanisms. However, AZT and ddNs such as d4T, ddI and ddA, which lack the 3'-OH moiety and are more lipophilic, may cross cell membranes mainly or partially by passive diffusion.[173,177-180] FLT has been shown to cross the cell membrane by two mechanisms, NBMPR-sensitive and NBMPR-insensitive processes.[333]

The importance of influx and efflux of ddNs, including AZT through membrane as initial determinants of their intracellular anabolism has been recognized. Moreover, it has been reported that dipyridamole (an inhibitor of nucleoside transport) potentiated the activity of AZT in monocyte/macrophage due at least in part to suppression of thymidine transport and phosphorylation, which resulted in increased phosphorylation of AZT.[334,335] On the other hand, coadministration of probenecid with AZT has provided elevated CSF/plasma ratios of AZT in both human and animals because of the inhibition of efflux of AZT from CSF.[210-212]

The objective of this study was to investigate the interaction of 5-halo-6-methoxy-5,6-dihydro analogs of AZT with the NBMPR-sensitive NT system, which is used by thymidine to cross human erythrocyte membranes. Due to the unavailability of radiolabeled 5,6-dihydro analogs which can be used for direct cellular uptake studies, thymidine influx competition experiments in which only radiolabeled thymidine is needed, were performed. Mouse erythrocytes were used as a simple model.

4.4.1. Thymidine influx time course in fresh murine erythrocytes.

The objective of this experiment was to determine the kinetic characteristics of thymidine influx across the cell membrane via the NBMPR-sensitive transport system in $B_6D_2F_1$ mouse erythrocytes at room temperature. It is known that metabolism of

thymidine does not occur in the mouse erythrocyte, so the erythrocyte provides an uncomplicated model for uptake studies.[306] It is also known that this transporter has a broad permeant specificity.[305,306,336,337]

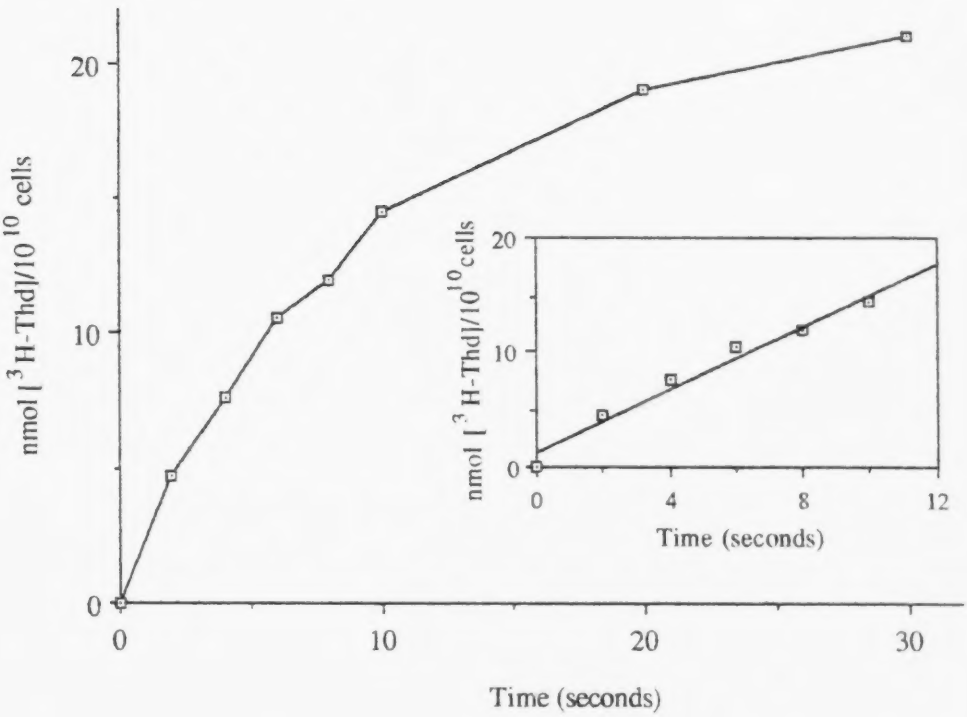


Figure 4.4.1. Time course of thymidine uptake in fresh murine erythrocytes. The intervals of thymidine uptake were initiated by the addition of 285 μM [^{3}H]-thymidine (0.2 mL) to equal volume of cell suspensions (3 x 10^8 cells). Fluxes intervals were terminated by the addition of NBMPR solution at a final concentration of 10 μM. The data shown are means of three replicate determinations.

The time course for thymidine influx was studied by incubating ^{3}H-thymidine with mouse erythrocytes, and stopping the transport process at specified time periods by the addition of an NBMPR solution. The transport rate was determined by measuring the intracellular concentration of ^{3}H-thymidine using the liquid scintillation method. The initial thymidine influx appears linear (see Figure 4.4.1.), but it became non-linear after 10 s. The proper use of NBMPR was essential in order to determine initial velocity of thymidine influx. In consideration of the technique of handling, a 3 s time interval was selected to determine the initial uptake rate for subsequent influx competition studies.

4.4.2. Determination of K_m and V_{max} for thymidine influx.

The kinetics of thymidine influx across mouse erythrocytes can be described by Michaelis-Menten kinetics,[338-340] in which the system can become saturable with thymidine. K_m is defined as the Michaelis constant, or half-saturation constant, and V_{max} is the maximum velocity. K_m and V_{max} values for the zero-trans influx of thymidine have always been measured in the absence of a competing permeant when each influx competition study was performed. Using the method of Hanes (Figure 4.4.2.),[307] the data plotted in Figure 4.4.3. provided a K_m = 0.076 mM, V_{max} = 5.81 nmoles/10^{10}cells/s. These values are comparable to the reported values (K_m = 0.075 mM, V_{max} = 6.25 nmoles/10^{10}cells/s).[341]

4.4.3. Determination of inhibition constants for AZT and its 5-halo-6-methoxy-5,6-dihydro analogs.

Although the NBMPR-sensitive NT system accepts a variety of compounds,[186] limitations on structural modifications tolerated by the transporter, especially on the sugar moiety, have been reported.[202,305,306,341] Decreased affinity has been demonstrated with dideoxythymidine suggesting that 3'-OH plays an important role in binding of pyrimidine nucleosides to the transporter. It has also been demonstrated that the replacement of 3'-

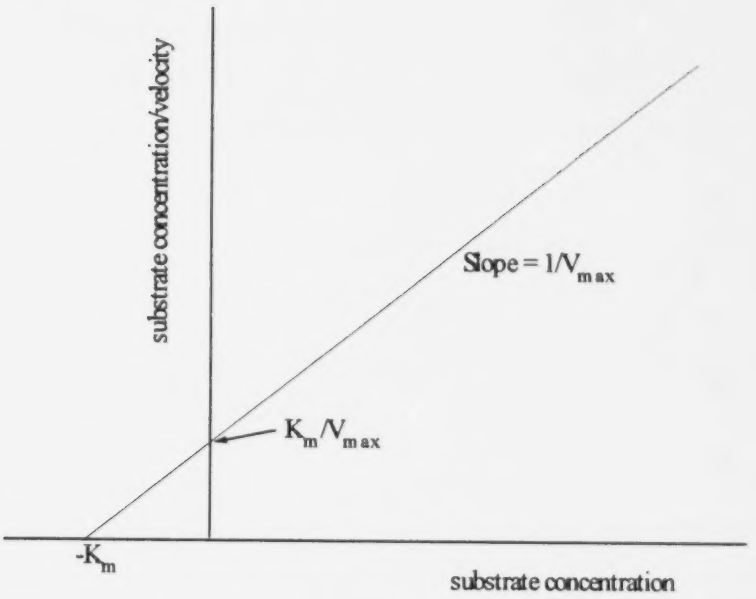


Figure 4.4.2. Hanes plot (method for determining K_m and V_{max}).

OH with a halogen is considerably less acceptable than replacement with a hydrogen. As reported previously, AZT and some other ddNs, such as d4T and ddA, cross cell membranes predominantly by simple diffusion due to the lack of a 3'-OH group and the higher lipophilicity.[173,177,306] However, the interaction between 5,6-dihydro uracil analogs and the NBMPR-sensitive nucleoside transport system has not been reported. Since thymidine transport inhibitors are known to enhance AZT anti-HIV activity,[342] we were prompted to investigate the interaction of 5-halo-6-methoxy-5,6-dihydro analogs of AZT with the thymidine transport system by performing zero-trans influx competition studies. The inhibition constant (K_i) can be obtained using the Dixon method[308] (Figure 4.4.4). An example is shown in Figure 4.4.5. For all the test compounds, the lines intersected above the [i]-axis, indicating a competitive inhibition of thymidine influx.[343] The value of K_i represents the relative affinity of the test compound for the external sites

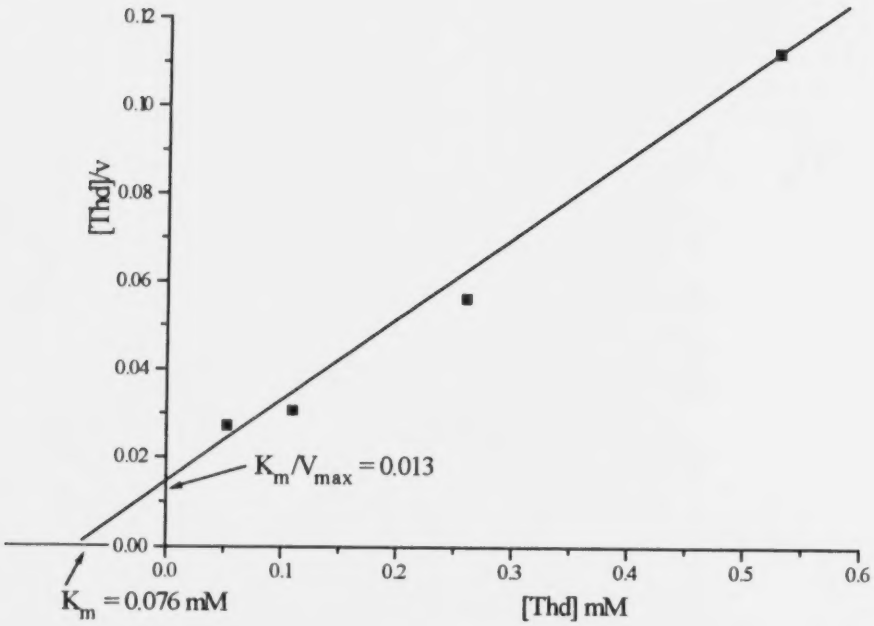


Figure 4.4.3. Determination of K_m and V_{max} for thymidine influx in fresh mouse erythrocytes. The influxes were measured in cell suspensions (3×10^8 cells) after 3 s of permeant exposure. The data shown are means of three replicate determinations.

on the NBMPR-sensitive nucleoside transport system. The larger the K_i value, the lower the affinity of the test compound for the transporter. The K_i values for AZT and its 5-halo-6-methoxy-5,6-dihydro analogs determined are presented in Table 4.4.1.

The K_i for AZT (see Table 4.4.1) was determined to be 1.33 mM, which is comparable to the previously reported value.[173] With increased lipophilicity that favors passive diffusion and the absence of a 3'-OH moiety which appeared to be an essential component for the transporter system,[305,306,341] less interaction would be expected from the 5,6-dihydro analogs of AZT. However, it was found that all of the trans- products behaved very similarly, with K_i values in the 0.2-0.4 mM range, indicating that these trans-

5,6-dihydro analogs have an increased affinity for the transporter relative to AZT. On the other hand, cis-(+)-(5S,6R)-AZTClOMe showed a significantly increased K_i value of greater than 1.5 mM, indicating a decreased affinity for the NT system due to unfavorable steric effects. This is likely because the trans- addition of halogen and methoxy to the 5,6-double bond renders the molecule more complimentary to the binding site since binding of the compound to the transport system is likely stereospecific.[204] With the increased affinity for the transporter observed with the trans- 5,6-dihydro analogs, there are two possible interpretations. One possibility is that these 5,6-dihydro analogs are actually transported by the transporter. The other possibility is that they are not transported, but that the decreased K_i value is just the result of non-specific lipophilic binding of the test compounds to the transport system. However, to resolve this problem, more experiments are needed. It is essential to do an efflux experiment to determine whether the NBMPR-sensitive NT system is involved in the membrane transport of these 5,6-dihydro analogs. In the efflux experiment, trans-acceleration of efflux provides evidence that the test compound is translocated across the membrane by the nucleoside transporter. Thus, the method we used does not provide direct information regarding the exact mechanism by which the test compound is transported. The important information obtained from this experiment is that all trans- addition products, regardless of the specific halogen atom, have a higher affinity relative to AZT for the NBMPR-sensitive thymidine transport system (increased interaction between these 5,6-dihydro analogs and the transport system), and the cis-(+)-(5S,6R)-AZTClOMe exhibited a decreased affinity for the same transport system. These results suggest that the configurations of the C^5 and C^6 chiral centers play an important role in determination of the affinity of 5,6-dihydro analogs for the mouse erythrocyte NBMPR-sensitive nucleoside transport system. Irrespective of whether the 5,6-dihydro analogs are transported by the NBMPR-sensitive transport system or not, the trans- 5,6-dihydro analogs do inhibit the transport of thymidine more effectively than AZT.

Table 4.4.1. Thymidine influx inhibition constants (K_i) for AZT and its 5-halo-6-methoxy-5,6-dihydro analogs in murine erythrocytes. Influxes of [^{3}H]-thymidine were measured in cell suspensions (3 x 10^8 cells) after 3 s of permeant exposure, in the absence or presence of graded concentrations of individual test compound. Influxes were initiated by the addition of the permeant solutions to the cell suspensions and terminated by the addition of NBMPR solution at a final concentration of 10 μM. The data shown are means from three experiments ± SD.

Compound	Inhibition constant (K_i)
AZT	1.33 ± 0.04 mM
cis-(+)-(5S,6R)-AZTClOMe	>> 1.5 mM
trans-(+)-(5R,6R)-AZTClOMe	0.36 ± 0.01 mM
trans-(+)-(5R,6R)-AZTBrOMe	0.45 ± 0.07 mM
trans-(-)-(5S,6S)-AZTBrOMe	0.50 ± 0.04 mM
trans-(+)-(5R,6R)-AZTIOMe	0.35 ± 0.03 mM
trans-(-)-(5S,6S)-AZTIOMe	0.20 ± 0.03 mM

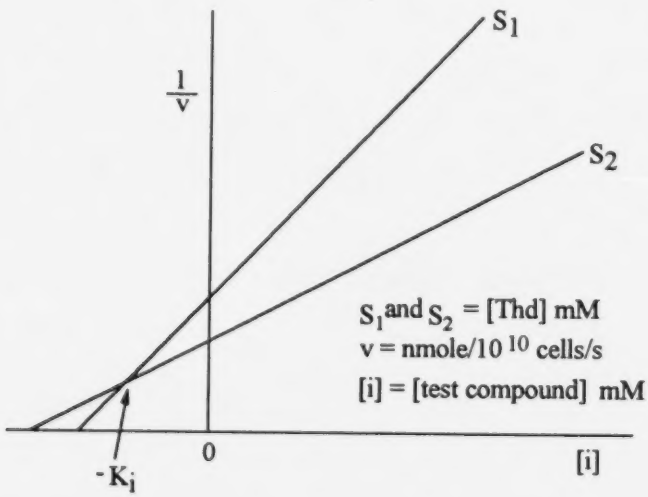


Figure 4.4.4. Dixon plot (method for determining K_i).

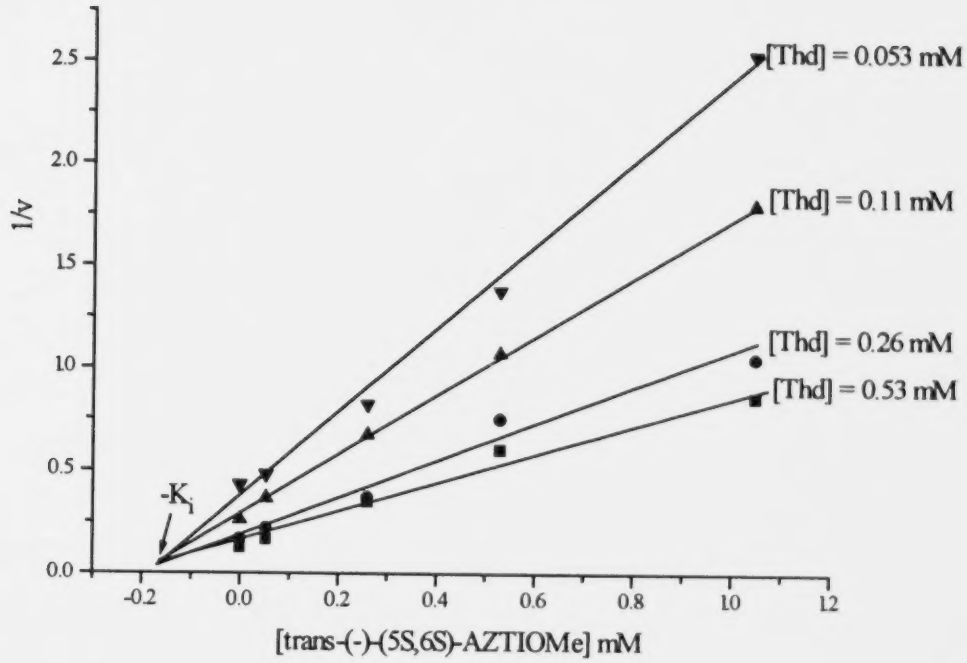


Figure 4.4.5. Determination of the K_i for trans-(-)-(5S,6S)-AZTIOMe. Influxes of [^{3}H]-thymidine were measured in fresh mouse erythrocytes (3 x 10^8 cells) after 3 s of permeant exposure, in the absence or presence of graded concentrations of trans-(-)-(5S,6S)-AZTIOMe. Influxes were initiated and terminated in response to metronome signals. NBMPR (20 μM) was used as the inhibitor to stop the influxes.

According to previous findings that a thymidine transport inhibitor increased the activity of AZT,[342] the 5,6-dihydro analogs we have studied are important. These nucleosides may exhibit double functions. They have better permeation ability because of their enhanced lipophilicity and on the other hand they reduce the permeation of thymidine into the cell because of their increased affinity for the NBMPR-sensitive transport system.

Although a number of AZT prodrugs (5'-esters) have been proposed, the interaction of those prodrugs with nucleoside transporters have not been reported.

4.5. *In vitro* stability of the 5-halo-6-methoxy 5,6-dihydro analogs in the presence of thymidine phosphorylase.

As an anti-cancer or anti-viral agent, a nucleoside must undergo anabolism to its 5'-triphosphate, and it is the latter which is responsible for its biological functions. However, for many nucleoside analogs, phosphorolysis (catabolic cleavage of the glycosidic bond with the formation of nucleobase and 1-phosphate ribose or 1-phosphate-2'-deoxy-ribose, shown as follows) is a competing process which causes degradation of the nucleoside.[344-347]

$$\text{Pyr-(d)R} + \text{Pi} \rightleftharpoons \text{Pyr} + \text{(d)-R-1-P}$$

Two pyrimidine nucleoside phosphorylases, uridine phosphorylase and thymidine phosphorylase, exist in the cytosol of mammalian cells and catalyze this reversible phosphorolysis of a number of naturally occurring and synthetic pyrimidine nucleosides. Thymidine phosphorylase is specific for the 2'-deoxyribosyl moiety of nucleosides, whereas uridine phosphorylase has a broader substrate specificity. Many pyrimidine nucleosides (e.g. uridine and 5-fluorouridine, FUDR) which do not bind to thymidine phosphorylase are good substrates for uridine phosphorylase.[344,348-351] 5-Substituted-2'-deoxyuridines are also better substrates for uridine phosphorylase than for thymidine phosphorylase. As an example, it has been found that the anabolism of FUDR (phosphorylation to corresponding 5'-phosphate-FUDR) is largely preempted by the rapid catabolism to FU followed by catabolic breakdown of the latter; this process is an important factor responsible for the decreased potency observed for FUDR *in vivo*.[344]

As prodrugs, it is important that the 5,6-dihydro analogs do not undergo catabolic cleavage, so that active drug concentrations can be attained (Figure 4.5.1.). Phosphorolysis of the prodrugs will result in a decrease in bioavailability, and stability towards phosphorolysis will allow the buildup the active drug *in vivo*.

Although a previous report indicated that AZT was not a substrate for either thymidine phosphorylase or uridine phosphorylase and that it only binds to uridine phosphorylase, d4T has been reported to be a good substrate of thymidine phosphorylase. On the basis of these data, it is postulated that the 5,6-dihydro analogs of d4T may provide greatly improved stability *in vivo*. Since AZT is already a poor substrate for phosphorolysis, the advantage of the 5,6-dihydro analogs may be less important. The susceptibility of the 5-halo-6-alkoxy-5,6-dihydro analogs of AZT, d4T and FLT for the phosphorolysis has not been reported. It was therefore considered important to investigate the stability of these prodrugs toward phosphorolysis.

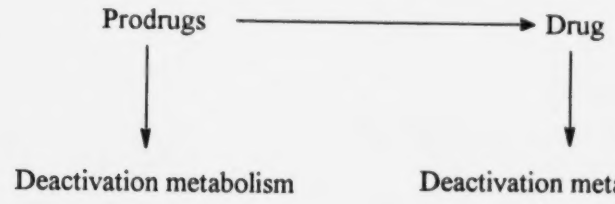


Figure 4.5.1. The possible metabolic pathways of the prodrugs.

An *in vitro* enzymatic study using *E. coli* thymidine phosphorylase provides a simple model for studying this process. *E. coli* thymidine phosphorylase is specific for pyrimidine deoxyribonucleosides.[310] Purine deoxyribonucleosides and ribonucleosides as well as deoxycytidine are not cleaved by this enzyme. The enzyme is stable for several months when kept properly. The stability of the enzyme is dependent on temperature and protein concentration. Thymidine phosphorylase from mammalian tissues has been reported to demonstrate similar substrate specificity to the bacterial enzyme.[348] This *in*

vitro phosphorolysis study was carried out by incubating the individual test compound with *E. coli* thymidine phosphorylase which is commercially available.

Thymidine was incubated with *E. coli* thymidine phosphorylase at 37°C and after specified time intervals (5, 10, 20, 30, 60 and 360 min), analysis was performed by HPLC to determine the degree of phosphorolysis. As illustrated in Figures 4.5.2., the process of phosphorolysis can be monitored by HPLC. The phosphorolysis of thymidine yielded thymine, which was confirmed by comparing the retention time of the products with that of a reference standard of thymine. The thymidine phosphorolysis time course is shown in Figure 4.5.3. It is clear that phosphorolysis reached its maximum after 10 min, and therefore 10 min was chosen for all phosphorolysis studies of AZT, d4T and their 5-halo-6-methoxy-5,6-dihydro analogs as well as other nucleoside analogs. The individual test compound (250 nmol) was incubated with thymidine phosphorylase (0.2 u) for 10 min at 37°C. The sample was analyzed by HPLC. The sensitivity of UV detection for the 5,6-dihydro analogs was approximately 1 μg/mL. As presented in Table 4.5.1., it was found that AZT does not undergo catabolic cleavage by *E. coli* thymidine phosphorylase. The 5-halo-6-methoxy-5,6-dihydro analogs of AZT have retained this stability. Other nucleoside analogs tested, including 2',3'-dideoxy-2',3'-didehydrothymidine (d4T), 2'-deoxyuridine (DU), and 5-ethyl-2'-deoxyuridine (EDU), underwent 10%, 57% and 72% phosphorolysis, respectively, whereas their 5-halo-6-methoxy (or azido)-5,6-dihydro analogs were found not to be susceptible to phosphorolysis by *E. coli* thymidine phosphorylase. The results suggest that the 5,6-disubstituted-5,6-dihydro pyrimidine analogs are not substrates for *E. coli* thymidine phosphorylase irrespective of whether their parent pyrimidines are, or are not, substrates for the enzyme. The stability against *E. coli* thymidine phosphorylase of the test compounds *in vitro* may suggest that they are stable in the blood circulation. However, the stability of these compounds upon incubation with uridine phosphorylase, which has a broader substrate specificity than thymidine phosphorylase, was not studied.

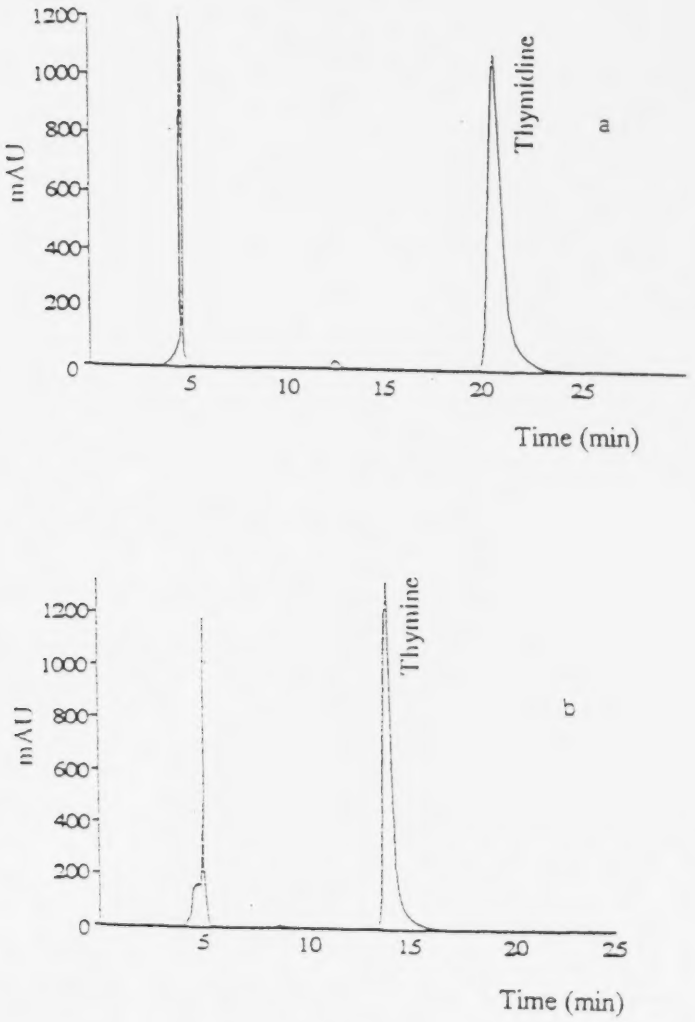


Figure 4.5.2. HPLC chromatograms for **a.** thymidine (retention time = 20 min) and **b.** thymine (retention time =14 min) using a Whatman partisil ODS column (25 cm) with water:methanol 9.5:0.5 at 2 mL/min as mobile phase and UV detection at 270 nm

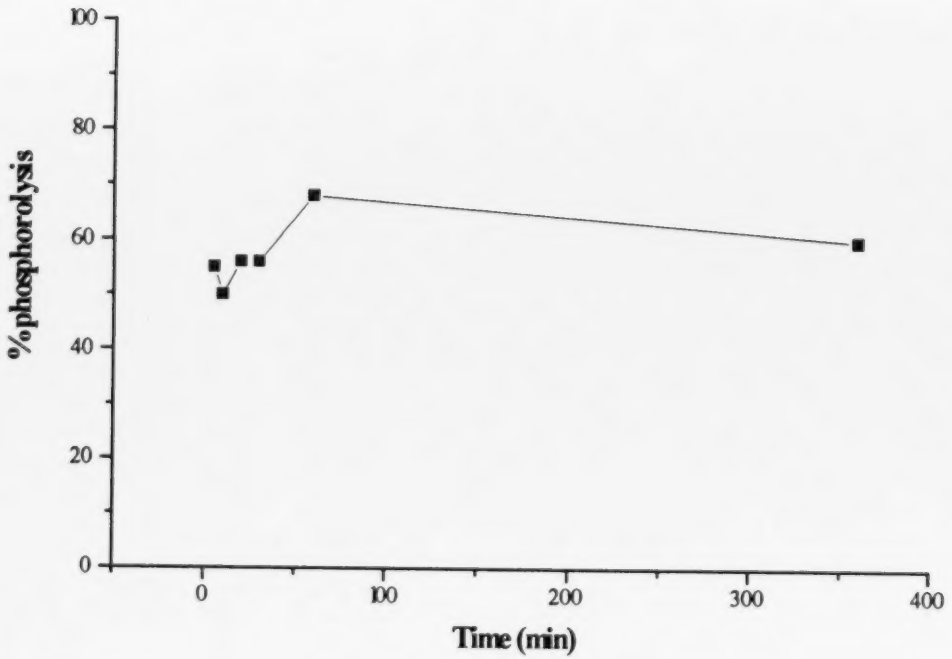


Figure 4.5.3. Thymidine phosphorolysis time course by *E. coli* thymidine phosphorylase at 37°C. Thymidine (250 nmole) was incubated with *E. coli* thymidine phosphorylase (0.2 u). The reaction was terminated by the addition of ice-cold methanol. The data shown are means from two replicate determinations.

Table 4.5.1. *In vitro* phosphorolysis of AZT, Thd, d4T, DU, EDU and their 5-halo-6-methoxy (or azido)-5,6-dihydro analogs by *E. coli* thymidine phosphorylase at 37°C. The phosphorolysis was determined at 10 min after the addition of the individual test compound (250 nmole) to *E. coli* thymidine phosphorylase (0.2 u). The reaction was terminated by the addition of ice-cold methanol. The data shown are means from n=3 for AZT and its 5,6-dihydro analogs. n=2 for the rest of the test compounds.

Compound	% Phosphorolysis
Azidothymidine (AZT)	0
cis-(+)-(5S,6R)-AZTClOMe	0
trans-(+)-(5R,6R)-AZTClOMe	0
trans-(+)-(5R,6R)-AZTBrOMe	0
trans-(-)-(5S,6S)-AZTBrOMe	0
trans-(+)-(5R,6R)-AZTIOMe	0
trans-(-)-(5S,6S)-AZTIOMe	0
Thymidine (Thd)	72
$ThdBrN_3$[a]	0
2',3'-Dideoxy-2',3'-didehydrothymidine (d4T)	10
$DDTBrN_3$[b]	0
cis-(+)-(5S,6R)-d4TClOMe	0
trans-(+)-(5R,6R)-d4TClOMe	0
trans-(+)-(5R,6R)-d4TBrOMe	0
trans-(-)-(5S,6S)-d4TBrOMe	0
trans-(+)-(5R,6R)-d4TIOMe	0
trans-(-)-(5S,6S)-d4TIOMe	0
2'-Deoxyuridine (DU)	57
$DUBrN_3$[c]	0
Edoxudine (5-ethyl-2'-deoxyuridine, EDU)	12
$EDUClN_3$[d]	0

[a] Mixture of trans-(+)-(5R,6R)- and trans-(-)-(5S,6S)-5-bromo-6-azido-5,6-dihydrothymidine.
[b] Mixture of trans-(+)-(5R,6R)- and trans-(-)-(5S,6S)-5-bromo-6-azido-5,6-dihydro-2',3'-dideoxy-2',3'-didehydrothymidine.
[c] Mixture of trans-(+)-(5R,6R)- and trans-(-)-(5S,6S)-5-bromo-6-azido-5,6-dihydro-2'-deoxyuridine.
[d] Mixture of trans-(+)-(5R,6R)- and trans-(-)-(5S,6S)-5-chloro-6-azido-5,6-dihydro-5-ethyl-2'-deoxyuridine.

4.6. Chemical stability of 5-halo-6-methoxy-5,6-dihydro analogs of AZT.

Chemical stability on standing is a prerequisite for any drug. Prodrugs and drugs must be sufficiently stable to have a reasonable shelf-life. However, the determination of shelf-life by accelerated studies is beyond the scope of this thesis. The chemical stability of 5-halo-6-methoxy-5,6-dihydro analogs of AZT, upon incubation with phosphate buffer, was therefore determined. Trans-(+)-(5R,6R)-5-chloro (bromo or iodo)-6-methoxy-5,6-dihydro analogs of AZT were incubated with phosphate buffer (pH 7.0, 0.06 M) at a concentration of 1 mM at 37°C for 24 h. The results, summarized in Table 4.6.1., indicate that all are stable for at least 24 h at 37°C. There was no sign of regeneration of AZT.

Table 4.6.1. The stability of 5,6-dihydro analogs upon incubation with phosphate buffer (pH 7.0, 0.06 M) at a concentration of 1 mM at 37°C.

Compound added	**5R,6R-(+)-AZTClOMe**		**5R,6R-(+)-AZTBrOMe**		**5R,6R-(+)-AZTIOMe**	
Compound measured	**% as prodrug**	**% AZT generated**	**% as prodrug**	**% AZT generated**	**% as prodrug**	**% AZT generated**
Time (h)						
0	99.54	0	99.02	0	95.08	0
1.0	99.33	0	99.11	0	99.75	0
3.0	99.06	0	98.76	0	90.57	0
5.0	99.59	0	99.23	0	98.44	0
10.0	99.34	0	99.07	0	97.36	0
24.0	99.47	0	99.21	0	99.76	0

4.7. *In vitro* regeneration of parent drugs from their 5-halo-6-methoxy-5,6-dihydro analogs.

In vitro regeneration of parent drugs from their 5-halo-6-methoxy-5,6-dihydro analogs was investigated in the presence of glutathione (GSH), mouse whole blood and the soluble enzyme fraction of mouse liver.

4.7.1. Conversion of the trans-(+)-(5R,6R)-5-bromo-6-methoxy-5,6-dihydro analog of AZT to AZT by glutathione.

The usefulness of a prodrug is dependent not only on its partition coefficient, transport characteristics and chemical stability, but also on its conversion to its active form. To be effective, prodrugs must convert to their active drug after administration since prodrugs are generally inactive.

The regeneration of uracils or uracil bases from their 5,6-dihydro analogs through thiol-mediated processes has been reported earlier.[318-323] 5-Bromo-6-methoxy-5,6-dihydrothymidine has been studied as a model compound to determine the mechanisms for its conversion to thymine by cysteine. When regeneration of uracils or uracil bases from their 5,6-dihydro analogs occurs via a thiol-mediated process, two mechanisms (E2 and SN2 as presented in Figure 4.7.1.) for the thiol-mediated dehalogenation are possible.[319] As shown in pathway A (E2 reaction), elimination of the C^5 halo substituent (X) involving a nucleophilic attack by glutathione (GSH) would give a carbanion or enolate anion (ii) as an intermediate. Alternatively, in pathway B (SN2 reaction), displacement of X by GSH to give (iii), followed by the subsequent attack of another molecule of GSH, would give the same intermediate (ii). Then elimination of methoxide anion from (ii) would yield parent nucleobase or nucleoside. However, the relative contributions of these mechanisms likely vary depending upon reactant concentrations, the nature of the halogen atom, and the substituents on the uracil ring system.[319] 5-Bromo-5-fluoro-5,6-dihydro-2'-

deoxyuridine has therefore been reported to act as a slow glutathione-mediated releaser of 5-fluoro-2'-deoxyuridine.[231] More recently, 5-halo-5-ethyl-6-methoxy-5,6-dihydro-2'-deoxyuridines have also been shown to be prodrugs of 5-ethyl-2'-deoxyuridine, with increased brain uptake and blood residence time.[232]

In this study, we attempted to investigate the conversion of the 5,6-dihydro prodrugs to their corresponding active drugs. First, the conversion of trans-(+)-(5R,6R)-5-bromo-6-methoxy-5,6-dihydro analog of AZT to AZT in the presence of varying amounts of glutathione was determined for specified reaction times. The results shown in Table 4.7.1. indicate that the conversion was dependent on the ratio of substrate:glutathione and was almost 100% when the substrate:glutathione molar ratio of 1:2 was used. In all cases the conversion was complete within 30 min.

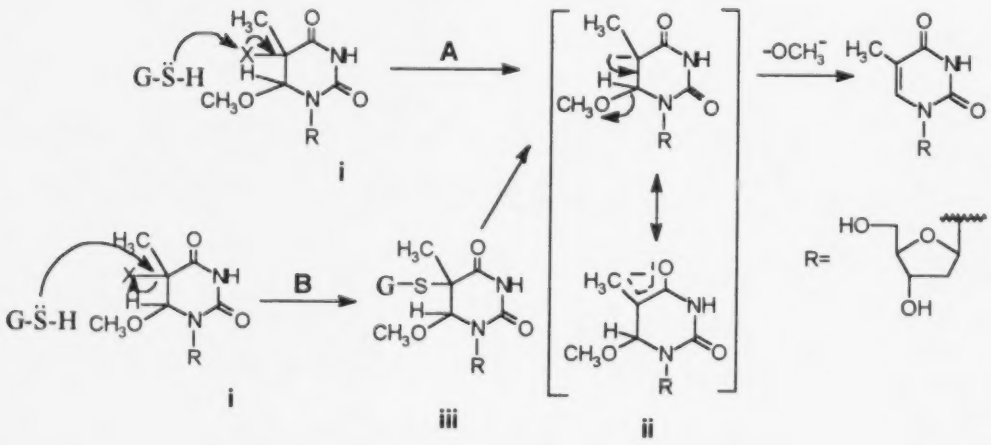


Figure 4.7.1. Putative mechanisms for the regeneration of uracil nucleoside or uracil nucleobase from their 5,6-dihydro analogs.[231]

Table 4.7.1. Regeneration of AZT from its trans-(+)-(5R,6R)-5-bromo-6-methoxy-5,6-dihydro analog by glutathione. Trans-(+)-(5R,6R)-AZTBrOMe was incubated with glutathione at different molar ratios for different time periods at 37°C. The results are from single determination only.

Molar ratio (substrate:glutathione)	**% regeneration of AZT**		
	30 min	60 min	90 min
1:0.2	11	8	9
1:2	98	99	ND[a]
1:10	100	ND[a]	ND[a]

a. ND = not determined.

The results suggest that the reaction is dependent upon the amount of glutathione and that 30 min is a sufficient reaction time for the reaction to go to completion. A substrate:glutathione molar ratio of 1:2, and a reaction time of 30 min was therefore selected for subsequent regeneration of parent drugs from corresponding 5,6-dihydro analogs by glutathione.

4.7.2. Regeneration of AZT, FLT and d4T from their corresponding 5-halo-6-methoxy-5,6-dihydro analogs by glutathione.

The chemical reactivity of glutathione and its potential involvement in the dehalogenation of the 5-halo-6-methoxy-5,6-dihydro analogs gives important insights into the more complicated *in vivo* process because of the presence of glutathione, cysteine and enzymes containing a thiol group.

Glutathione is an important substance with immune-enhancing and antioxidant properties. Extracellular glutathione protects cells against oxidation injury and is a major

transport form of the amino acid cysteine. Within cells, glutathione functions as an antioxidant. Glutathione is also believed to be important in the initiation and progression of lymphocyte activation, and thus essential for host defense. The normal glutathione level in plasma is 5.99 μM, of which 5.71 μM is in the form of reduced glutathione. In mammalian tissues, the glutathione concentration is in the range of 0.5-10 mM, whereas the cysteine concentration is in the range of 0.03-0.1 mM.[352] However, the level of glutathione in HIV seropositive group is about one third of that in normal people.[353]

It was of interest to design prodrugs which would provide optimal regeneration of active drugs in AIDS patients and provide a sustained release of active drug *in vivo*. Based on the mechanisms proposed, the thiol-mediated dehalogenation process is dependent on the nature of the halogen species, the steric effect and electronegativity of different halogens, as demonstrated by Sander *et al*.[322] In their studies, 5-FU was resistant to dehalogenation, while 5-chloro-, 5-bromo- and 5-iodo- uracils underwent dehalogenation. Another study reported by Duschinsky *et al* showed regeneration of the 5,6-double bond for various 5,6-disubstituted 5-fluorodihydrouracils.[231] It was found that 5-chloro-6-alkoxy-5,6-dihydro-5-fluorouridines did not convert to FUDR, while 5-bromo-6-alkoxy-5,6-dihydro-5-fluorouridines converted at different rates. It has also been shown that the % regeneration of FUDR from 5-bromo-6-alkoxy-5,6-dihydro-5-fluorouridines is slightly decreased upon the elongation of the chain at C^6 in the rank of $-OCH_3 > -OC_2H_5 > -OC_3H_7$. In addition, the conversion is dependent on the substituents on the uracil ring system.[231,322]

The regeneration of AZT, FLT and d4T from their 5-halo-6-alkoxy-5,6-dihydro analogs was therefore investigated. The individual test compound was incubated with glutathione using a substrate:glutathione molar ratio of 1:2 at 37°C based on the results in Table 4.7.1. After specified time periods, the sample was analyzed by HPLC. The results are summarized in Tables 4.7.2.-4.7.4. The regeneration of the parent drugs from their 5,6-dihydro analogs were found to be dependent on the nature of the halogen at the C^5

position. In general, both iodo- and bromo- analogs converted to their parent drugs in the presence of glutathione with a relative % regeneration of -I > -Br, while chloro analogs were stable (no conversion was observed). The results suggest that when the halogen at C^5 position is changed from chloro to bromo or iodo, this susceptibility to attack by glutathione is increased dramatically due to the fact that bromine and iodine are better leaving groups than chlorine. Conversion of 5-halo-6-alkoxy-5,6-dihydro analogs that have alkoxy groups other than the methoxy group was not studied. Based on the mechanisms proposed in Figure 4.7.1., the difference in conversion rate from two trans-diastereomers [trans-(+)-(5R,6R)- and trans-(-)-(5S,6S)-] should not be significant because of the similar electronic and steric effects for both isomers. However, the difference between cis- and trans- isomers is dependent on which mechanism is more involved. If the E2 mechanism plays a more important role, the conversion from the cis-isomer should be slower than from the trans- isomer, because it presents a bigger steric hindrance to the approach of the nucleophile than trans- isomer does. In contrast, if SN2 is more involved, the conversion from the trans- isomer will be slower than that from the cis- isomer, because the attack of the nucleophile is from the other side of the halogen and the cis- isomer would exhibit a smaller steric hindrance relative to the trans- isomer. However, a large difference was observed between the trans-(-)-(5S,6S)-FLTIOMe (90%) and the trans-(+)-(5R,6R)-FLTIOMe (50%) isomers. Trans-(-)-(5S,6S)-d4TBrOMe (50%) and trans-(+)-(5R,6R)-d4TBrOMe (85%) also showed different % regenerations.

Table 4.7.2. Regeneration of AZT from its 5-halo-6-methoxy-5,6-dihydro analogs by glutathione. The individual test compound was incubated with glutathione at a molar ratio (substrate:glutathione) of 1:2 for specified time period at 37°C. The results are means of two determinations.

Substrate	Molar ratio (substrate:glutathione)	Incubation time	% Conversion
trans-(+)-(5R,6R)-AZTIOMe	1:2	30 min	100
trans-(-)-(5S,6S)-AZTIOMe	1:2	30 min	100
trans-(+)-(5R,6R)-AZTBrOMe	1:2	30 min	98
trans-(-)-(5S,6S)-AZTBrOMe	1:2	30 min	90
cis-(+)-(5S,6R)-AZTClOMe	1:2	24 h	0
trans-(+)-(5R,6R)-AZTClOMe	1:2	24 h	0

Table 4.7.3. Regeneration of FLT from its 5-halo-6-methoxy-5,6-dihydro analogs by glutathione.[315] The individual test compound was incubated with glutathione at a molar ratio (substrate:glutathione) of 1:2 for specified time period at 37°C. The results are means of two determinations.

Substrate	Molar ratio (substrate:glutathione)	Incubation time	% Conversion
trans-(+)-(5R,6R)-FLTIOMe	1:2	30 min	50
trans-(-)-(5S,6S)-FLTIOMe	1:2	30 min	90
trans-(+)-(5R,6R)-FLTBrOMe	1:2	30 min	10
trans-(-)-(5S,6S)-FLTBrOMe	1:2	30 min	10
cis-(+)-(5S,6R)-FLTClOMe	1:2	30 min	0
trans-(+)-(5R,6R)-FLTClOMe	1:2	30 min	0

Table 4.7.4. Regeneration of d4T from its 5-halo-6-methoxy-5,6-dihydro analogs by glutathione. The individual test compound was incubated with glutathione at a molar ratio (substrate:glutathione) of 1:2 for specified time period at 37°C. The results are means of two determinations.

Substrate	Molar ratio (substrate:glutathione)	Incubation time	% Conversion
trans-(+)-(5R,6R)-d4TIOMe	1:2	30 min	>95
trans-(-)-(5S,6S)-d4TIOMe	1:2	30 min	>95
trans-(+)-(5R,6R)-d4TBrOMe	1:2	30 min	85
trans-(-)-(5S,6S)-d4TBrOMe	1:2	30 min	50
cis-(+)-(5S,6R)-d4TClOMe	1:2	21 h	0
trans-(+)-(5R,6R)-d4TClOMe	1:2	21 h	0

Compared to 5,6-dihydro-5-fluorouridines[231] such as the 5-bromo-6-alkoxy analogs which showed only 18-21% conversion to the parent compound after 24 h, the 5-bromo (or iodo)-6-methoxy-5,6-dihydro analogs studied in this thesis convert to their parent compound much more completely (50-100% after 30 min). The results support previous findings that the conversion from 5,6-dihydro analogs is dependent on the specific substituents on the uracil ring. The % regeneration of parent nucleosides from their corresponding 5,6-dihydro analogs was AZT > d4T > FLT.

4.7.3. Regeneration of AZT from its 5-halo-6-methoxy-5,6-dihydro analogs in mouse blood.

The results from the glutathione study indicated that the regeneration of AZT, FLT or d4T from their 5-halo-6-methoxy-5,6-dihydro analogs by glutathione was dependent upon the nature of the halogen atom, being easily regenerated from their 5-iodo (or

bromo) analogs, but not from the chloro analogs. A similar relationship is postulated to occur in blood due to the presence of GSH, cysteine or enzymes containing a thiol moiety. It has been shown that the 5-bromo-6-methoxy-5-ethyl-5,6-dihydro analog of EDU is converted to EDU in both blood and plasma, and that the conversion in blood (58%) was more extensive than in plasma (8%), suggesting that blood cells play an important role in this conversion.[232]

In vitro studies of 5-halo-6-methoxy-5,6-dihydro analogs were conducted using mouse whole blood. The regeneration of AZT *in vitro* in mouse blood more closely represents the biological environment the prodrugs will experience than incubation with GSH. The advantage of the *in vitro* mouse blood study over an *in vivo* study is that other systemic effects which would complicate analysis of the data can be avoided.

Regeneration of AZT from its 5-halo-6-methoxy-5,6-dihydro analogs in mouse blood was investigated by incubating 100 μL of heparinized Balb/c mouse blood with 100 μg of the individual test compound for 10 min. The sample was then analyzed by HPLC. The results, presented in Table 4.7.5., suggest that regeneration of AZT is again dependent upon the nature of the halogen atom at the C^5 position, where the % regeneration order is I > Br > Cl. After a 10 min incubation, 5-iodo-6-methoxy-5,6-dihydro analogs of AZT were completely converted and 5-bromo-6-methoxy-5,6-dihydro analogs were partially converted to AZT. Furthermore, 5-chloro-6-methoxy-5,6-dihydro analogs were not converted at all. The trans-(+)-(5R,6R)- and trans-(-)-(5S,6S)-5-bromo-6-methoxy-5,6-dihydro analogs of AZT showed slightly different amounts of conversion.

Table 4.7.5. The regeneration of AZT from its 5-halo-6-methoxy-5,6-dihydro analogs in mouse blood. The individual test compound was incubated with mouse whole blood for 10 min at 37°C. The data are from single determination only.

Compound	Regeneration %
trans-(+)-(5R,6R)-AZTIOMe	100
trans-(-)-(5S,6S)-AZTIOMe	100
trans-(+)-(5R,6R)-AZTBrOMe	59
trans-(-)-(5S,6S)-AZTBrOMe	88
cis-(+)-(5S,6R)-AZTClOMe	0
trans-(+)-(5R,6R)-AZTClOMe	0

4.7.4. Regeneration of AZT and d4T from their 5-halo-6-methoxy-5,6-dihydro analogs in the soluble enzyme fraction of mouse liver.

Liver is rich in enzymes and is an important site of drug metabolism. The regeneration of AZT and d4T from their 5-halo-6-methoxy-5,6-dihydro analogs were examined by incubation of the test compound with a soluble liver enzyme fraction for 30 min. The sample was then analyzed by HPLC. The results are listed in Tables 4.7.6 and 4.7.7.

Again it was found that the regeneration of AZT or d4T from their 5,6-dihydro analogs is dependent on the nature of the halogen at C^5 and that 5-iodo (or bromo)-6-methoxy-5,6-dihydro analogs converted to their corresponding nucleoside analogs while 5-chloro-6-methoxy-5,6-dihydro analogs did not convert under the same conditions.

Table 4.7.6. The regeneration of AZT from its 5-halo-6-methoxy-5,6-dihydro analogs in a soluble enzyme fraction of mouse liver. The individual test compound was incubated with the soluble enzyme fraction of mouse liver for 30 min at 37°C. The data shown are from single determination only.

Compound	Regeneration%
trans-(+)-(5R,6R)-AZTIOMe	80
trans-(-)-(5S,6S)-AZTIOMe	95
trans-(+)-(5R,6R)-AZTBrOMe	26
trans-(-)-(5S,6S)-AZTBrOMe	43
cis-(+)-(5S,6R)-AZTClOMe	0
trans-(+)-(5R,6R)-AZTClOMe	0

Table 4.7.7. The regeneration of d4T from its 5-halo-6-methoxy-5,6-dihydro analogs in a soluble enzyme fraction of mouse liver. The individual test compound was incubated with the soluble enzyme fraction of mouse liver for 30 min at 37°C. The data shown are from single determination only.

Compound	Regeneration%
trans-(+)-(5R,6R)-d4TIOMe	85
trans-(-)-(5S,6S)-d4TIOMe	95
trans-(+)-(5R,6R)-d4TBrOMe	75
trans-(-)-(5S,6S)-d4TBrOMe	52
cis-(+)-(5S,6R)-d4TClOMe	0
trans-(+)-(5R,6R)-d4TClOMe	0

4.8. Blood levels of AZT in mice obtained after i.v. injection of AZT or its 5-halo-6-methoxy-5,6-dihydro analogs.

An *in vivo* study using Balb/c mice was conducted by i.v. injection of AZT and its 5-halo-6-methoxy-5,6-dihydro analogs (144 μmol/kg) via the tail vein. The concentrations of AZT and the individual 5,6-dihydro analog in plasma were determined by HPLC analysis after extraction by Sep-Pak cartridges. However, the presence of the 5,6-dihydro analogs after administration of the 5-bromo and 5-iodo-6-methoxy-5,6-dihydro analogs was not detectable, which is due to the limited sensitivity (approximately 1μg/mL) of HPLC-UV detection and their fast regeneration to AZT. AZT was therefore quantitated, using FIAU as an internal standard. The recovery of AZT and FIAU from the Sep-Pak was 88±5% and 84±4%, respectively. In the case of the 5-chloro-6-methoxy-5,6-dihydro analog of AZT (AZTClOMe), quantitation of either AZT or AZTClOMe after i.v. injection of AZTClOMe was problematic due to the low quantity of AZT generated from AZTClOMe, and the low UV absorbance of AZTClOMe. The dose of trans-(+)-(5R,6R)-AZTClOMe was therefore increased to 240 μmol/kg. Both AZT and AZTClOMe were then detected using FLTBrOMe as an internal standard. The blood levels of AZT in Balb/c mice after i.v. injection of AZT, trans-(+)-(5R,6R)-AZTBrOMe, trans-(-)-(5S,6S)-AZTBrOMe, trans-(+)-5R,6R)-AZTIOMe or trans-(-)-(5S,6S)-AZTIOMe (144 μmol/kg) are presented in Table 4.8.1. and Figure 4.8.1. These results demonstrate that the level of AZT in blood is similar after injection of the same molar dose of either AZT or its 5-iodo (or bromo)-6-methoxy-5,6-dihydro analogs. The concentrations of both AZT and trans-(+)-AZTClOMe after administration of trans-(+)-AZTClOMe are shown in Table 4.8.2.

The *in vivo* results suggest that both AZTBrOMe and AZTIOMe are rapidly converted to AZT, thereby providing similar blood levels of AZT as those observed after

Table 4.8.1. Blood levels of AZT after i.v. administration of AZT and its 5-bromo (or iodo)-6-methoxy-5,6-dihydro analogs (144 μmol/kg). The data shown are means ± SD, n=3.

Compound administered	AZT	5R,6R-(+)-AZTBrOMe	5S,6S-(-)-AZTBrOMe	5R,6R-(+)-AZTIOMe	5S,6S-(-)-AZTIOMe
Compound measured	AZT (μg/mL)	AZT (μg/mL)	AZT (μg/mL)	AZT (μg/mL)	AZT (μg/mL)
Time (min)					
5	37.50±5.87	20.9±2.4	37.67±5.90	38.00±5.01	35.80±7.23
15	23.37±8.31	9.51±1.27	21.63±3.46	16.06±7.60	11.50±1.32
30	8.72±0.72	6.70±1.15	10.98±6.08	11.70±5.00	7.77±1.16
45	6.38±1.83	2.70±0.67	9.40±6.77	5.75±0.90	ND
60	2.85±1.12	2.00±0.89	2.20±0.66	3.60±1.64	0.65±0.07
90	NDT	1.30±0.56	1.23±0.53	1.65±0.40	2.00±0.00
120		NDT	0.53±0.08	1.23±0.49	1.80±0.85
150			NDT	NDT	1.50±0.26

ND = not determined.

NDT = not detectable.

AZT administration. However the % regeneration of AZT from AZTClOMe was very low, resulting in a relatively low concentration of AZT (less than 1/10 of that obtained from the 5-iodo (or bromo)-6-methoxy-5,6-dihydro analogs).

This study again demonstrated the impact of the halogen at C^5 in the 5,6-dihydro analogs on the regeneration of AZT. Both 5-bromo and iodo analogs resulted in a similar % regeneration of AZT, which was much larger than for the 5-chloro analog.

A compound with a moderate % regeneration is needed to provide a sustained release of AZT. Based on the two mechanisms proposed (shown in Figure 4.7.1, page 109) and previous reports that the dehalogenation processes were dependent on both substituents at C^5 and C^6 positions,[231-233,318-323] it is postulated that alkoxy moiety at C^6 in 5,6-dihydro analogs of AZT should have an impact on the rate and %conversion of 5,6-dihydro analogs of AZT to AZT in addition to the impact observed from the halogen

Table 4.8.2. Analysis results of blood samples after i.v. injection of trans-(+)-(5R,6R)-5-chloro-6-methoxy-5,6-dihydro analog of AZT (240 μmol/kg). The data shown are means from n=2.

Time	15 min	30 min	60 min	90 min
AZT concentration (μg/mL blood)	2.5	2.5	1.3	NDT
AZTClOMe concentration (μg/mL blood)	28.8	12.5	16.3	1.1

NDT = not detectable.

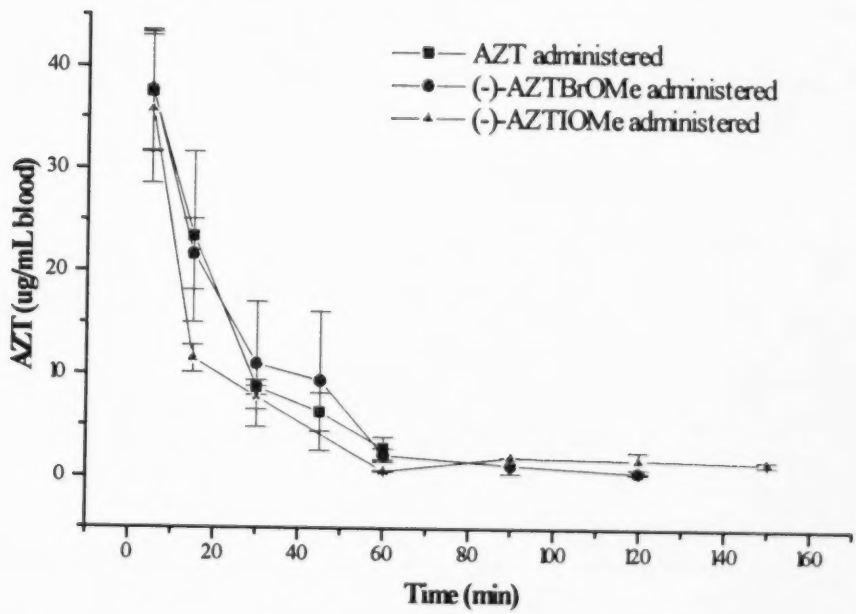


Figure 4.8.1. The blood levels of AZT obtained after i.v. injection of AZT (144 μmol/kg) and its trans-(-)-(5S,6S)-5-bromo(or iodo)-6-methoxy-5,6-dihydro analogs (equimolar concentration), n=3.

at the C^5 position. Therefore, a 5-halo-6-alkoxy-5,6-dihydro analog of AZT with a moderate conversion rate and % conversion to AZT may be designed by an appropriate combination of substituents at C^5 and C^6. The study involving compounds that possess different alkoxy groups at the C^6 position is under investigation by others in our research group. However, this study was done in normal mice. The regeneration of AZT from the 5-halo-6-alkoxy-5,6-dihydro analogs may be a rate-limiting process in infected patients if the regeneration of AZT is a thiol-mediated process since the glutathione level in HIV infected subjects is lower than in uninfected individuals. A dosage of 200 mg (0.75 mmole) of AZT every 8 h is now more commonly used. If an equimolar concentration of the 5,6-dihydro analog is given, 1.5 mmole glutathione is needed to convert all of the prodrug (5,6-dihydro analog) molecules to AZT. However glutathione (5.99 μM in plasma) is not the only substance that exists in the body that is involved in thiol-mediated reactions. For example, cysteine (0.03-0.1 mM in mammalian tissues) and enzymes containing a thiol moiety are also available for this regeneration.

As discussed earlier, many ester prodrugs of AZT have been proposed, including DP-AZT and 5'-aliphalic acid-AZTs. Although DP-AZT was designed to target in brain, the concentration of AZT in plasma is constantly lower (50%) than that achieved after AZT itself due to the fast clearance of DP-AZT from blood. 5'-Aliphatic acid-AZTs have been the most promising in producing a sustained release of AZT in plasma to date.[205] AZT concentrations in plasma after 5'-aliphatic acid-AZTs by intraperitoneal administration were shown to be more constant and persistent, especially when the acyl chain is increased to C18 as a result of longer retention due to its significantly increased lipophilicity and sustained enzymatic hydrolysis.

4.9. Biodistribution for [2-^{14}C]-AZT and [2-^{14}C]-5-bromo-6-methoxy (or ethoxy)-5,6-dihydro analogs of AZT in mice.

Our primary objective is to develop prodrugs of nucleoside RT inhibitors that will have high brain uptake. The biodistribution studies in mice were performed to determine if the enhanced lipophilicity of the 5,6-dihydro analogs resulted in an increased brain uptake.

The *in vivo* distribution study of [2-^{14}C]-AZT, [2-^{14}C]-trans-(+)-(5R,6R)-AZTBrOMe, [2-^{14}C]-trans-(-)-(5S,6S)-AZTBrOMe and [2-^{14}C]-trans-(+)-(5R,6R)-AZTBrOEt after i.v. injection via the tail vein was carried out in conventional Balb/c mice. The amount of radioactivity in blood, brain, bone, heart, liver, spleen, lung, kidney, bladder and urine at several time intervals was determined using a combustion/liquid scintillation counting method. The combustion efficiency ranged between 84-97%. The results presented in Tables 4.9.1.- 4.9.4. are percentage of the administered radioactivity recovered per g or per mL of tissue, i.e., ID%/g or mL tissue (ID=injected dose). The results for urine samples were based on the small volume of urine excreted, calculated per mL urine sample. The number greater than 100% is due to the fact that a small volume (< 1 mL) was actually excreted. The data suggest that the test compounds (both AZT and its 5,6-dihydro analogs) are distributed to all of the tissues examined. The extensive distribution of the test compounds into various tissues, particularly at very early time periods, is likely due to their high lipophilicity. The high level of radioactivity in kidney, bladder and urine suggests that renal clearance is a major excretion pathway for these compounds.

These experiments have provided very exciting findings which include an increased concentration in brain and prolonged blood levels, together with no increase in bone after injection of [2-^{14}C]-trans-(+)-(5R,6R)-AZTBrOMe, [2-^{14}C]-trans-(-)-(5S,6S)-AZTBrOMe or [2-^{14}C]-trans-(+)-(5R,6R)-AZTBrOEt as compared to an equimolar dose

of [2-^{14}C]-AZT. Although AZT has been approved for the treatment of AIDS since 1987, its bone marrow toxicity, limited brain uptake and poor blood kinetic characteristics have challenged both clinicians and researchers involved in the treatment of AIDS patients and in the development of more efficient anti-AIDS compounds, respectively. To reduce bone marrow toxicity, lower doses of AZT have been recommended,[354] but this may result in lower brain levels, thereby decreasing AZT's therapeutic effectiveness for children and for cerebral infection.[19] Many types of prodrugs have been made in an attempt to overcome this problem. To date, all efforts have resulted in failure to produce a prodrug with a clear-cut therapeutic advantage. Among the prodrugs reported in the literature, the DP-AZT appears to be the most successful, producing significant increases in brain delivery in animal studies.[257] DP-AZT resulted in a three-fold-higher concentration of AZT in rat brain, and a two-fold increase in concentrations in the CSF was observed in dog studies.[257] However, the biodistribution of DP-AZT was not examined. Since a lower concentration of AZT is found in plasma after DP-AZT dosing, it is therefore not known if DP-AZT will provide a clinical advantage. The 5,6-dihydro analogs investigated in this study provided a three-fold increase in brain uptake in mice, which is similar to the result obtained from DP-AZT.

Although the *in vivo* conversion of the 5-bromo (or 5-iodo)-6-methoxy-5,6-dihydro analogs to the parent compound was very fast, as shown in Section 4.8., no significant differences in blood concentrations of AZT after administration of AZT or its 5-bromo (or 5-iodo)-6-methoxy-5,6-dihydro analogs, was observed within 2 h post injection. The biodistribution study did show an improvement with respect to brain targeting and in blood at longer time periods after 2 h (Tables 4.9.1.-4.9.4.).

Table 4.9.1. Distribution of radioactivity in mice after tail vein injection of [2-^{14}C]-AZT (2 μCi, specific activity 56 mCi/mmole). Data shown are means ± SD, n=3

Tissue	10 min	30 min	60 min	120 min	240 min	360 min
Blood[1]	4.15±0.21	1.48±0.29	0.54±0.13	0.09±0.03	0.03±0.03	0.03±0.02
Heart[2]	4.43±0.34	1.52±0.35	0.65±0.16	0.13±0.03	0.02±0.01	Trace
Liver[2]	5.31±0.58	1.81±0.38	0.73±0.15	0.18±0.05	0.06±0.03	Trace
Spleen[2]	4.94±0.02	2.15±0.83	1.07±0.21	0.54±0.17	0.09±0.07	0.05±0.03
Lung[2]	5.01±0.52	1.74±0.33	0.67±0.17	0.12±0.05	0.03±0.01	Trace
Bone[2]	3.48±0.23	1.48±0.47	0.67±0.13	0.27±0.06	0.28±0.24	0.03±0.01
Brain[2]	0.35±0.03	0.16±0.04	0.10±0.02	0.04±0.01	0.03±0.01	Trace
Kidney[2]	14.7±44.0	4.14±0.65	3.35±1.63	0.31±0.20	0.03±0.01	Trace
Bladder[2]	17.50±4.14	11.19±5.73	14.09±12.17	5.53±8.60	8.33±10.29	Trace
Urine[1]	312±98	1203±654	877±338	259±352	62±33	5.61±6.60

[1] The number is the percentage of the administered radioactivity recovered per mL of sample.

[2] The number is the percentage of the administered radioactivity recovered per g of tissue.

Table 4.9.2. Distribution of radioactivity in mice after tail vein injection of [2-^{14}C]-trans-(-)-(5S,6S)-AZTBrOMe (2 μCi, specific activity 56 mCi/mmole). Data shown are means ± SD, n=3.

Tissue	10 min	30 min	60 min	90 min	120 min	210 min	360 min	480 min
Blood[1]	1.05±0.03	1.05±0.15	0.90±0.15	0.91±0.20	0.75±0.17	0.75±0.04	0.93±0.04	0.84±0.19
Heart[2]	4.24±0.58	1.08±0.16	0.71±0.15	0.43±0.06	0.29±0.07	0.23±0.08	0.34±0.02	0.28±0.02
Liver[2]	5.79±0.62	1.23±0.38	0.71±0.24	0.60±0.25	0.23±0.01	0.23±0.02	0.25±0.07	0.19±0.03
Spleen[2]	5.16±1.23	1.24±0.36	1.22±0.28	1.05±0.41	0.42±0.03	0.30±0.07	0.30±0.02	0.28±0.04
Lung[2]	4.71±1.15	1.16±0.29	0.74±0.14	0.45±0.20	0.37±0.09	0.37±0.04	0.29±0.13	0.32±0.03
Bone[2]	3.24±0.24	1.83±0.35	0.49±0.11	0.40±0.12	0.34±0.19	0.13±0.01	0.11±0.01	0.11±0.01
Brain[2]	0.69±0.17	0.30±0.12	0.15±0.01	0.13±0.04	0.12±0.04	0.02±0.01	0.05±0.01	0.05±0.02
Kidney[2]	16.97±4.16	1.97±1.03	1.11±0.18	0.56±0.06	0.40±0.02	0.21±0.03	0.22±0.02	0.20±0.02
Bladder[2]	29.14±11.12	41.15±30.39	11.49±8.53	8.62±7.78	1.95	0.61±0.32	0.27±0.02	0.27±0.04
Urine[1]	292±158	ND	259±113	268±84	ND	9.27±4.44	3.17±1.11	0.94±0.04

[1] The number is the percentage of the administered radioactivity recovered per mL of sample.

[2] The number is the percentage of the administered radioactivity recovered per g of tissue.

ND = not determined.

Table 4.9.3. Distribution of radioactivity in mice after tail vein injection of [2-^{14}C]-trans-(+)-(5R,6R)-AZTBrOMe (2 μCi, specific activity 56 mCi/mmole). Data shown are means ± SD, n=3.

Tissue	10 min	30 min	60 min	120 min	240 min	360 min
Blood[1]	3.42±0.51	1.48±0.32	1.32±0.17	0.77±0.15	0.88±0.07	0.67±0.16
Heart[2]	3.22±0.48	1.45±0.19	0.76±0.06	0.38±0.05	0.27±0.03	0.21±0.05
Liver[2]	4.17±0.50	1.49±0.34	0.83±0.20	0.23±0.02	0.17±0.02	0.16±0.05
Spleen[2]	3.43±0.35	1.95±0.69	1.59±0.60	0.44±0.04	0.24±0.01	0.18±0.06
Lung[2]	3.64±0.39	1.57±0.10	1.09±0.56	0.38±0.01	0.30±0.02	0.25±0.06
Bone[2]	2.32±0.57	1.01±0.16	0.89±0.19	0.20±0.02	0.10±0.01	0.06±0.01
Brain[2]	1.06±0.18	0.48±0.08	0.38±0.23	0.11±0.01	0.07±0.01	0.04±0.01
Kidney[2]	10.04±1.19	4.56±1.26	1.94±0.58	0.31±0.05	0.18±0.01	0.13±0.03
Bladder[2]	25.70±11.82	64.66±19.76	68.05±51.16	4.33±2.66	0.50±0.27	0.34±0.22
Urine[1]	818±877	835±93	800±432	55±16	8.2±2.05	8.38±6.85

[1] The number is the percentage of the administered radioactivity recovered per mL of sample.

[2] The number is the percentage of the administered radioactivity recovered per g of tissue.

Table 4.9.4. Distribution of radioactivity in mice after tail vein injection of [2-^{14}C]-trans-(+)-(5R,6R)-AZTBrOEt (2 μCi, specific activity 56 mCi/mmole). Data shown are means ± SD, n=3.

Tissue	10 min	30 min	60 min	120 min	240 min	360 min	480 min
Blood[1]	4.14±0.86	2.20±0.89	0.98±0.25	0.76±0.05	0.64±0.09	0.60±0.07	0.55±0.18
Heart[2]	4.21±0.30	1.64±0.64	0.52±0.03	0.29±0.01	0.18±0.18	0.17±0.03	0.15±0.04
Liver[2]	8.53±2.64	2.47±0.97	0.85±0.09	0.45±0.06	0.29±0.06	0.20±0.02	0.19±0.06
Spleen[2]	4.97±0.45	1.98±0.98	0.85±0.13	0.91±0.13	0.30±0.03	0.19±0.01	0.17±0.04
Lung[2]	4.85±0.29	1.94±0.72	0.71±0.05	0.40±0.02	0.28±0.15	0.24±0.02	0.21±0.07
Bone[2]	2.78±0.94	1.79±1.14	0.66±0.24	0.35±0.01	0.16±0.02	0.09±0.01	0.08±0.02
Brain[2]	1.14±0.12	0.41±0.12	0.21±0.02	0.16±0.03	0.10±0.01	0.06±0.01	0.05±0.01
Kidney[2]	15.18±1.23	4.78±1.47	1.39±0.25	0.43±0.02	0.62±0.15	0.18±0.02	0.17±0.05
Bladder[2]	63.74±41.26	32.40±12.61	24.42±24.82	11.78±11.10	0.32±0.03	Trace	Trace
Urine[1]	774±376	467±47	359±170	462±186	45.31±14.35	1.84±0.81	11.98±8.20

[1] The number is the percentage of the administered radioactivity recovered per mL of sample.

[2] The number is the percentage of the administered radioactivity recovered per g tissue.

4.9.1. Comparison of blood radioactivity levels in mice after i.v. injection of [2-^{14}C]-AZT, [2-^{14}C]-trans-(+)-(5R,6R)-AZTBrOMe or [2-^{14}C]-trans-(+)-(5R,6R)-AZTBrOEt.

Since the antiviral activity is believed to be due to AZT-triphosphate (AZT-TP), an adequate concentration of AZT-TP should be maintained to achieve the anticipated anti-AIDS effect. Although intracellular AZT-TP has a half-life of 3 to 4 h, the half-life of AZT is only about 1 h and the correlation between intracellular AZT-TP and AZT is not clear.[122] The current therapy using AZT requires frequent dosing. On the other hand, to avoid the undesirable side effects, especially dose-limiting bone marrow toxicity, lower concentrations are preferred.[118] The occurrence of severe anemia and neutropenia in patients receiving a dose of 1500 mg/day is significantly higher than that with 500 mg/day (29% vs. 5%). Another study showed that AZT was discontinued earlier in more subjects in standard treatment (250 mg every 4 hour) than in a lower dose (100 mg every 4 h) group (40% vs. 29%).[354] The same study also demonstrated that the lower dose is as effective as the standard dose. To balance the two factors (adequate concentration for efficacy and lower concentration for reduced toxicity), the prodrug approach has become one of the most researched methods. Prodrugs which have a longer residence time in body and provide a sustained release of the parent drug at concentrations adequate for its anti-HIV activity, but not causing toxicity, are needed. There have been extensive efforts to reduce AZT's clearance from blood.[205-207] The majority of the prodrugs of nucleoside RT inhibitors investigated todate are their 5'-derivatives. Among them, 5'-aliphatic acid-AZTs showed promising results in mice.[205] Caprate and stearate AZT analogs in this series demonstrated a sustained release in plasma. However, more studies are needed for further evaluation. The compounds (5'-aliphatic acid esters) showed a dramatic increase in lipophilicity and have log P values up to greater than 5 in comparison with AZT (log P = –0.31) in a chloroform:phosphate buffer system. As was discussed earlier, a compound with excessive lipid solubility will no longer circulate in the bloodstream, and therefore can

not exhibit its biological activity. The biodistribution of these previously unknown 5,6-dihydro compounds has obviously not been reported.

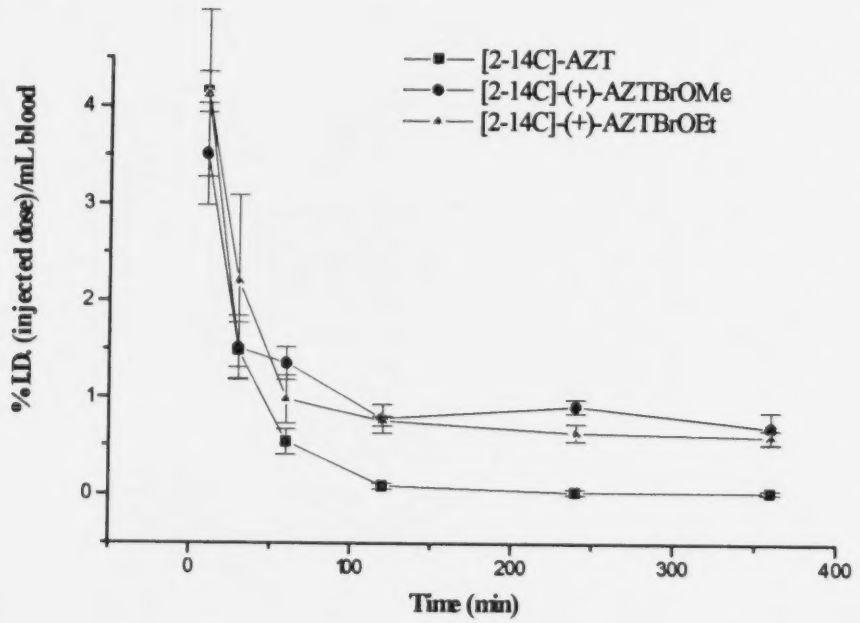


Figure 4.9.1. Clearance of radioactivity from blood after i.v. injection of [2-^{14}C]-AZT and [2-^{14}C]-5-bromo-6-alkoxy-5,6-dihydro analogs of AZT (2 μCi, specific activity 56 mCi/mmole). Data shown are means ± SD, n=3.

Radioactivity determined in blood up to 6 h after i.v. injection of [2-^{14}C]-AZT, [2-^{14}C]-trans-(+)-(5R,6R)-AZTBrOMe and [2-^{14}C]-trans-(+)-(5R,6R)-AZTBrOEt expressed as % I.D./mL blood is plotted as shown in Figure 4.9.1. It was found that radioactivity levels from the 5,6-dihydro analogs were significantly higher than those from AZT after 2 h (student T test, $P \leq 0.05$), suggesting that the decline of radioactivity in blood after dosing with the 5-bromo-6-alkoxy (methoxy or ethoxy)-5,6-dihydro analogs is

slower than that of AZT and the difference between blood levels of radioactivity after doses of trans-(+)-(5R,6R)-AZTBrOMe and trans-(+)-(5R,6R)-AZTBrOEt is not significant. The AUCs (area under the curve) are calculated by a traperoidal method and presented in Table 4.9.5. Trans-(+)-(5R,6R)-AZTBrOMe and trans-(+)-(5R,6R)-AZTBrOEt have exhibited significantly increased AUCs relative to that after AZT (346 and 322 vs. 116). In this experiment, only total ratioactivity was measured and the chemical species involved is not completely known. However, based on previous findings regarding the metabolism of AZT, and the results described in this thesis, possible chemical species associated with the radioactivity measured are shown in Figure 4.9.2. The results obtained from this study show that 5,6-dihydro analogs have an increased retention time in the body. The slower clearance of the 5,6-dihydro analogs from the body is likely due to their increased lipophilicity. Greater retention is expected for 5,6-dihydro analogs that have alkoxy groups larger than ethoxy at C^6, because the longer chain at C^6 will likely increase lipophilicity and these analogs should undergo conversion to AZT more slowly.

Table 4.9.5. The AUCs of radioactivity in blood after i.v. injection of ^{14}C-AZT, ^{14}C-trans-(+)-(5R,6R)-AZTBrOMe and ^{14}C-trans-(+)-(5R,6R)-AZTBrOEt in mice.

Compound administered	**[2-^{14}C]-AZT**	**[2-^{14}C]-(5R,6R)-AZTBrOMe**	**[2-^{14}C]-(5R,6R)-AZTBrOEt**
$AUC_{10\text{-}360\ min}$(%ID·min/g)[a]	116	346	322

[a]AUC: Area under the curve which represents the percentage of the administered dose per mL of blood versus time.

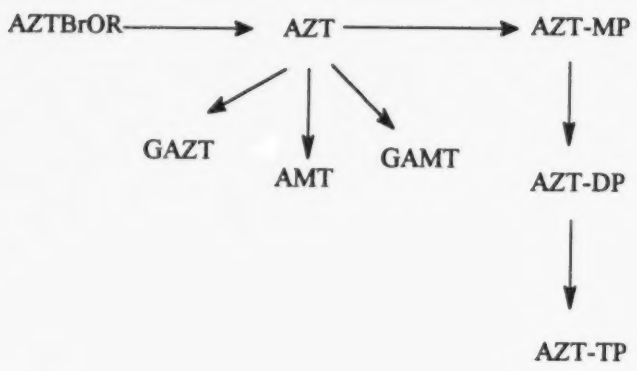


Figure 4.9.2. The postulated metabolic pathways for AZTBrOR.

4.9.2. Comparison of brain radioactivity levels in mice after i.v. injection of [2-^{14}C]-AZT, [2-^{14}C]-trans-(+)-(5R,6R)-AZTBrOMe or [2-^{14}C]-trans-(+)-(5R,6R)-AZTBrOEt.

Neurological disorders are common in AIDS patients and these disorders contribute to the morbidity of patients in advanced stages of infection with HIV. Isolation of the HIV virus from cerebrospinal fluid and brain tissue has demonstrated the presence of HIV infection in the CNS.[41,124,130-132] Although administration of AZT improves the CNS dysfunction clinically, the penetration of AZT into the brain is believed to be minimal, as demonstrated in rat studies.[134,135] Brain/serum concentration ratios for AZT in mice after administration of 50 mg/kg, and 250 mg/kg, have been reported to be dose dependent, the ratios being higher after doses of 250 mg/kg (brain/serum = 0.283) than those after doses of 50 mg/kg (brain/serum = 0.064). However, due to AZT's dose-related bone marrow toxicity, it is not recommended to increase brain uptake by increasing the administered dose. Moreover, it has been shown that bulk efflux of AZT from CSF and brain via the organic anion transport system plays an important role in the observed limited AZT levels in CSF and brain. These findings underscore the need for antiviral agents, including prodrugs, that can more effectively penetrate the BBB and provide

elevated concentrations of AZT-TP. Various prodrugs of AZT have therefore been designed.

The novel compounds studied in this thesis were designed with the objective of increasing lipophilicity. AZT has been reported to cross cell membranes by passive diffusion although the organic anion transport system mediates the efflux of AZT from CSF and brain and it is known that passive diffusion is increased when lipophilicity is increased. As was discussed earlier, log P's between 0.9 and 2.5 are assumed to be the optimal range for certain radiopharmaceuticals.[355] The 5,6-dihydro analogs of AZT discussed in this thesis have log P values in this range; the exception is cis-(+)-(5S,6R)-AZTClOMe, which has a log P of about 0.5. Therefore a higher delivery to the brain was expected. Indeed, analysis of mouse brain samples showed that the brain radioactivity levels after [2-^{14}C]-5-bromo-6-alkoxy (methoxy or ethoxy)-5,6-dihydro-3'-azido-3'-deoxythymidines (2 μCi, specific activity 56 mCi/mmole) were significantly higher than those after AZT (2 μCi, specific activity 56 mCi/mmole) ($P \leq 0.05$, Figure 4.9.3.). At 10 min after i.v. injection, the radioactivity level in brain after administration of the 5,6-dihydro analogs of AZT was three times greater than that after AZT. The ratio of brain/blood radioactivity at 10 min after administration of [2-^{14}C]-AZT was found to be about 0.08. The ratio increased to the 0.28 - 0.65 range for the 5,6-dihydro analogs, suggesting that the 5,6-dihydro analogs have a higher brain uptake due to their increased lipophilicity. Over the range of time periods studied (10 min to 8 h), the radioactivity in brain declined. This is likely due to bi-directional diffusion of AZT regenerated from the prodrug. The AUCs and MRTs (mean residence times) are summarized in Table 4.9.6. The AUCs of the dihydro analogs studied were significantly larger than that for AZT (approximately three-fold). The MRT for the 5-bromo-6-ethoxy-5,6-dihydro analog was significantly increased, although the MRT for the 5-bromo-6-methoxy-5,6-dihydro analog is almost same as that for AZT. This is likely due to a further increase in brain uptake of the 6-ethoxy analog, or slower conversion to AZT. Again only total radioactivity was

determined and the chemical species are not completely understood. The results obtained can be explained by the model depicted in Figure 4.9.4. The prodrug (5,6-dihydro analog) of AZT enters brain more readily because of its enhanced lipophilicity relative to AZT, although regeneration of AZT takes place at the same time. After the prodrug is inside the brain, it can be gradually converted to AZT followed by the formation of AZT-MP, AZT-DP and AZT-TP which are then trapped in the brain because of their greater hydrophilicity. AZT generated either outside or inside the brain will be subject to efflux via the organic anion transport system out of the brain.

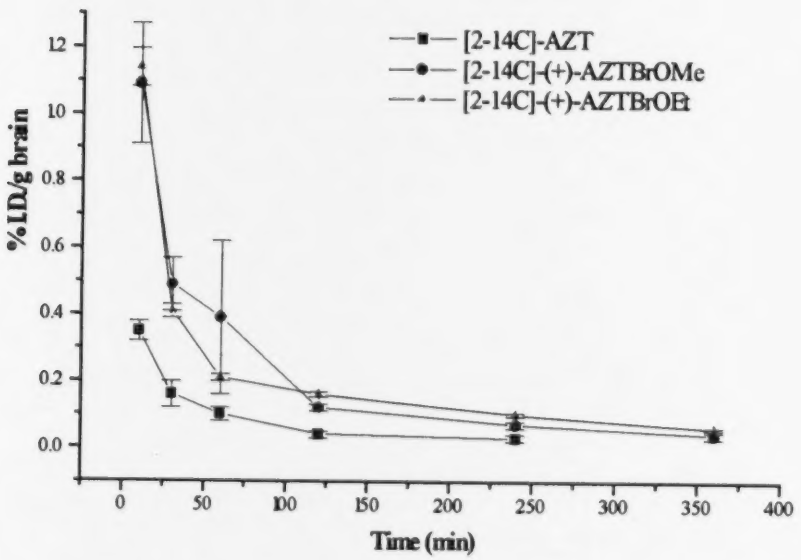


Figure 4.9.3. Brain levels of radioactivity after i.v. injection of [2-^{14}C]-AZT and [2-^{14}C]-5-bromo-6-alkoxy-5,6-dihydro-3'-azido-3'-deoxythymidines (2 μCi, specific activity 56 mCi/mmole. The data shown are means ± SD, n=3.

Table. 4.9.6. Comparison of radiopharmacokinetic parameters.

Compound administered	[2-^{14}C]-AZT	[2-^{14}C]-Trans-(+)-(5R,6R)-AZTBrOMe	[2-^{14}C]-Trans-(+)-(5R,6R)-AZTBrOEt
$AUC_{0-\infty}$ (%ID·min/g)[a]	17.4	53.8	51.5
Mean residence time (min)	131.5	141.2	236.3

[a]AUC: Area under the curve which represents the percentage of the administered dose per g of brain versus time.

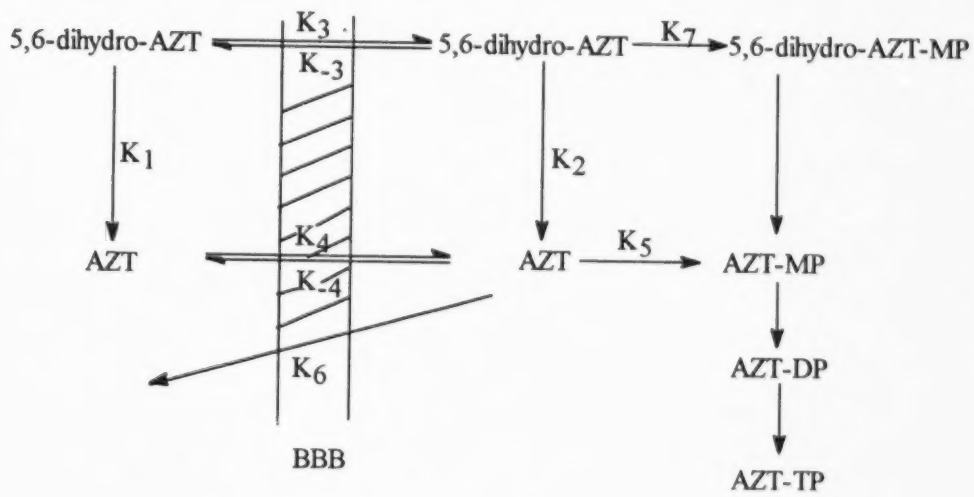


Figure 4.9.4. Diagram of the proposed uptake process for AZT in the brain.

If one assumes that K_1=K_2, K_3=K_{-3} and K_4=K_{-4}, then the net trapping effect will depend on the relative magnitude of K_5 and K_6, with K_5 leading to accumulation and K_6 leading to decreased concentrations. The net balance will also be influenced by K_7, if this reaction were to occur and by any influence that the prodrug might exert on the organic anion transporter (K_6).

The elevated brain uptake after dosing with the 5,6-dihydro analogs suggests that the net effects lead to accumulation and better therapeutic efficacy is to be expected by utilizing these AZT analogs, but an analysis of levels of ^{14}C-AZT-TP formed from the prodrugs is required for clarification of this situation.

The 5,6-dihydro analogs designed have provided a similar increase in brain uptake of radioactivity to DP-AZT. However, the glycosyl phosphotriester prodrug described earlier provides a higher brain concentration, resulting in a ratio of brain/serum of the prodrug greater than 1 with a significantly improved pharmacokinetic profile of AZT-5'-phosphate in brain compared to that for AZT (peak concentration of 156 nmol/g vs. 5 nmol/g; half-life 24 h vs. 1 h; AUC 4366 nmol·h/g vs. 4 nmol.h/g). Although phosphotriesters provide an increased brain uptake, they are more toxic than AZT in CEM-C113 cells. The clinical potential of these types of compounds remains to be determined.

4.9.3. Comparison of bone radioactivity levels in mice after i.v. injection of [2-^{14}C]-AZT, [2-^{14}C]-trans-(+)-(5R,6R)-AZTBrOMe or [2-^{14}C]-trans-(+)-(5R,6R)-AZTBrOEt.

Bone marrow toxicity is the main side effect resulting from the clinical use of AZT. It is believed to be the result of formation of AMT and a deficiency of dTTP caused by AZT. To reduce the toxicity associated with AZT, the level of AZT in bone marrow has to be minimized. It was important to examine whether the increased brain uptake and decreased clearance observed for 5-halo-6-methoxy-5,6-dihydro analogs of AZT are associated with elevated bone uptake.

Analysis of bone samples showed that the quantities of radioactivity in bone following the administration of AZT, or these 5,6-dihydro analogs of AZT, were about the same (Figure 4.8.5.), suggesting that the 5,6-dihydro analogs do not increase the bone concentration of AZT. Because of the slower clearance and higher brain uptake observed,

if smaller or less frequent doses of the 5,6-dihydro analogs to provide the same therapeutic effect, less toxicity will be expected. In addition, development of resistance is expected to be delayed by smaller doses of AZT since it was reported that resistance tends to develop earlier with AZT dosages of 1200 to 1500 mg/day than dosages of 500 mg/day.

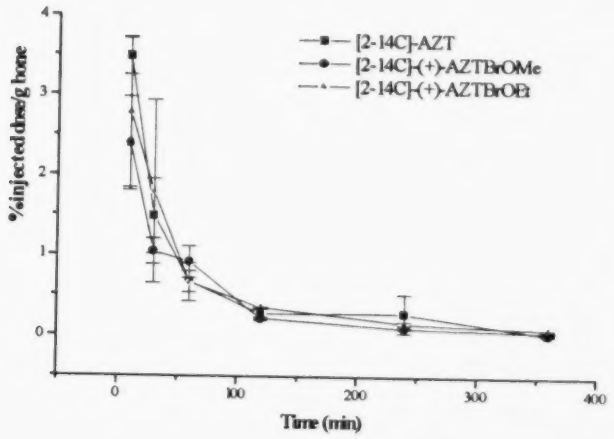


Figure 4.9.5. Bone levels of radioactivity after i.v. injection of [2-^{14}C]-AZT and [2-^{14}C]-5-bromo-6-alkoxy-5,6-dihydro analogs of AZT (2 μCi, specific activity = 56 mCi/mmol). The data shown are means ± SD, n=3.

4.10. Brain subcellular distribution of radioactivity after administration of [2-^{14}C]- AZT or its [2-^{14}C]- 5-bromo-6-alkoxy-5,6-dihydro analogs.

The biodistribution studies described in Section 4.9. suggest that 5-bromo-6-methoxy (or ethoxy)-5,6-dihydro-3'-azido-3'-deoxythymidines were more effective than AZT for brain targeting, since about three times more radioactivity was trapped in brain tissue 10 min after injection of these prodrugs than for AZT. However, the results obtained from the biodistribution study do not unambiguously prove the presence of AZT, its 5,6-dihydro prodrugs or their metabolites in the cerebral tissue. Penetration of the

nerve terminals is presumably important in the actions of drugs such as AZT. A brain subcellular fractionation study was therefore performed to investigate whether the higher brain uptake of radioactivity resulting from the prodrugs was associated with specific binding of the 5-bromo-6-methoxy (or ethoxy)-5,6-dihydro-3'-azido-3'-deoxythymidines to any specific subcellular components, particularly the P_2 fraction which contains synaptosomes (pinched-off nerve endings). An *ex vivo* study was performed in mice, using literature methods with minor modifications.[311]

4.10.1. Preparation of mouse brain subcellular fractions and analysis of protein.

Four to six mouse brains were combined and subjected to a sequence of centrifugations as illustrated in the Experimental Section (page 75). The P_1, P_2 and P_3 pellets were obtained after 1,000 x *g*, 10,000 x *g* and 100,000 x *g* centrifugations, respectively. The S_3 fraction was the final supernatant. The protein content of each fraction was determined by the Lowry method and the results, defined as the amount of protein in each fraction divided by the total amount of protein, are shown in Figure 4.10.1. The P_1 fraction, which contains nuclei and cell debris contained 13.7% protein. The P_2 fraction, which is a mixture of myelin fragments, synaptosomes and mitochondria, was found to represent 49.3% of the total protein. The P_3 fraction, which is the microsomal fraction, contained 12.0% protein and the S_3 fraction, which is the soluble fraction, contained 25.1% protein. These results indicated that about half of the total brain protein was recovered from the P_2 fraction. The protein contents in subcellular fractions P_1, P_2, P_3, and S_3 from guinea pig brain were reported to be 30, 33, 14 and 23%, respectively.[311] The differences in P_1 and P_2 between our results and the reported results are possibly due to the species difference or the degree of homogenization. The more the brain tissue is homogenated, the less is expected in this fraction, since the P_1 contains nuclei and cell debris.

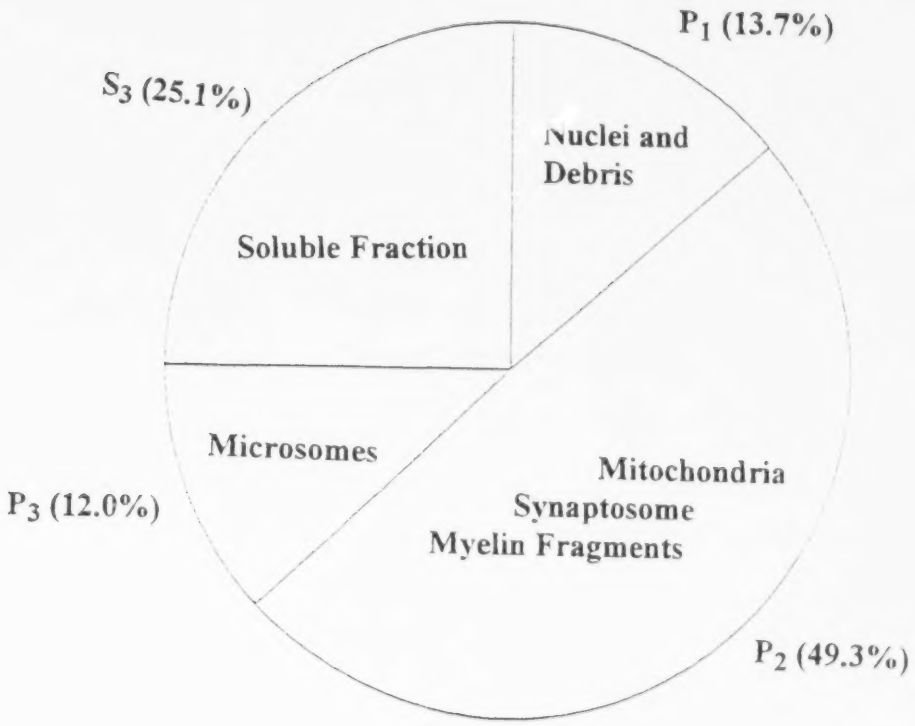


Figure 4.10.1. Protein distribution in mouse brain subcellular fractions.

4.10.2. Subcellular distribution of [2-^{14}C]-AZT and its [2-^{14}C]-5-bromo-6-alkoxy-5,6-dihydro analogs in brain after jugular vein injection.

The subcellular distributions of [2-^{14}C]-AZT, [2-^{14}C]-trans-(+)-(5R,6R)-AZTBrOMe, and [2-^{14}C]-trans-(+)-(5R,6R)-AZTBrOEt in brain were investigated in Balb/c mice after injecting the compound via the jugular vein. Distribution studies after tail vein injection of [2-^{14}C]-AZT (2 μCi, specific activity 56 mCi/mmole), [2-^{14}C]-trans-(+)-(5R,6R)-AZTBrOMe (2 μCi, specific activity 56 mCi/mmole), and [2-^{14}C]-trans-(+)-(5R,6R)-AZTBrOEt (2 μCi, specific activity 56 mCi/mmole) have indicated that less than 5% of the injected dose was present in blood after 10 min (see Tables 4.9.1.-4.9.4.).

Jugular vein injections were expected to increase the amount delivered to the brain. For this study, the brains from 5 mice were pooled prior to fractionation to increase the accuracy of the analysis. The mouse brains were collected 10 min after the jugular vein injection and fractionated into P_1, P_2, P_3 and S_3 fractions by the centrifugation method. The quantity of radioactivity and protein content were determined for each fraction. The distribution of radioactivity as well as RSC (relative specific concentration) for the compounds investigated are presented in Figure 4.10.2. and Table 4.10.1. A value of RSC greater than 1 represents a considerable localization.

The results obtained indicate that the subcellular distribution of radioactivity in the brain after jugular vein injection of [2-^{14}C]-AZT and its [2-^{14}C]-5-bromo-6-methoxy (or ethoxy)-5,6-dihydro analogs was very similar. There was a considerable amount of radioactivity in the S_3 fraction, suggesting that there was no specific binding of the test compounds to any subcellular component in P_1, P_2, or P_3 or that the binding was not very "tight". Although a similar brain subcellular distribution pattern in brain was observed after [2-^{14}C]-AZT and its [2-^{14}C]-5-bromo-6-methoxy (or ethoxy)-5,6-dihydro analogs (see column B in Table 4.10.1.), higher radioactivity levels were recovered from brain after [2-^{14}C]-5-bromo-6-methoxy (or ethoxy)-5,6-dihydro analogs of AZT than after [2-^{14}C]-AZT, which suggested that the 5-bromo-6-alkoxy-5,6-dihydro analogs of AZT enter brain at a faster rate than AZT.

Table 4.10.1. Distribution of radioactivity after administration of [^{14}C]-AZT and [^{14}C]-trans-(+)-(5R,6R)-5-bromo-6-methoxy (or ethoxy)-5,6-dihydro analogs of AZT in subcellular fractions of mouse brain (pooled samples from 5 mice).

	Protein distribution (A)[a]			**Radioactivity distribution (B)[b]**			**CPM/mg protein (C)[c]**			**Relative Specific Concentration (RSC=B/A)**		
	AZT	**AZTBrOMe**	**AZTBrOEt**	**AZT**	**AZTBrOMe**	**AZTBrOEt**	**AZT**	**AZTBrOMe**	**AZTBrOEt**	**AZT**	**AZTBrOMe**	**AZTBrOEt**
P_1	15.9%	13.3%	11.9%	14.6%	4.6%	5.2%	110	208	390	0.92	0.34	0.44
P_2	51.7%	53.1%	43.0%	7.6%	5.6%	7.7%	28	64	159	0.15	0.11	0.18
P_3	9.6%	8.7%	17.6%	3.4%	1.4%	2.4%	47	97	119	0.35	0.16	0.14
S_3	22.8%	24.9%	27.5%	74.5%	88.4%	84.7%	1000	2145	2735	3.30	3.55	3.10

[a]% total protein recovered.

[b]% total radioactivity recovered.

[c]radioactivity (CPM)/mg protein.

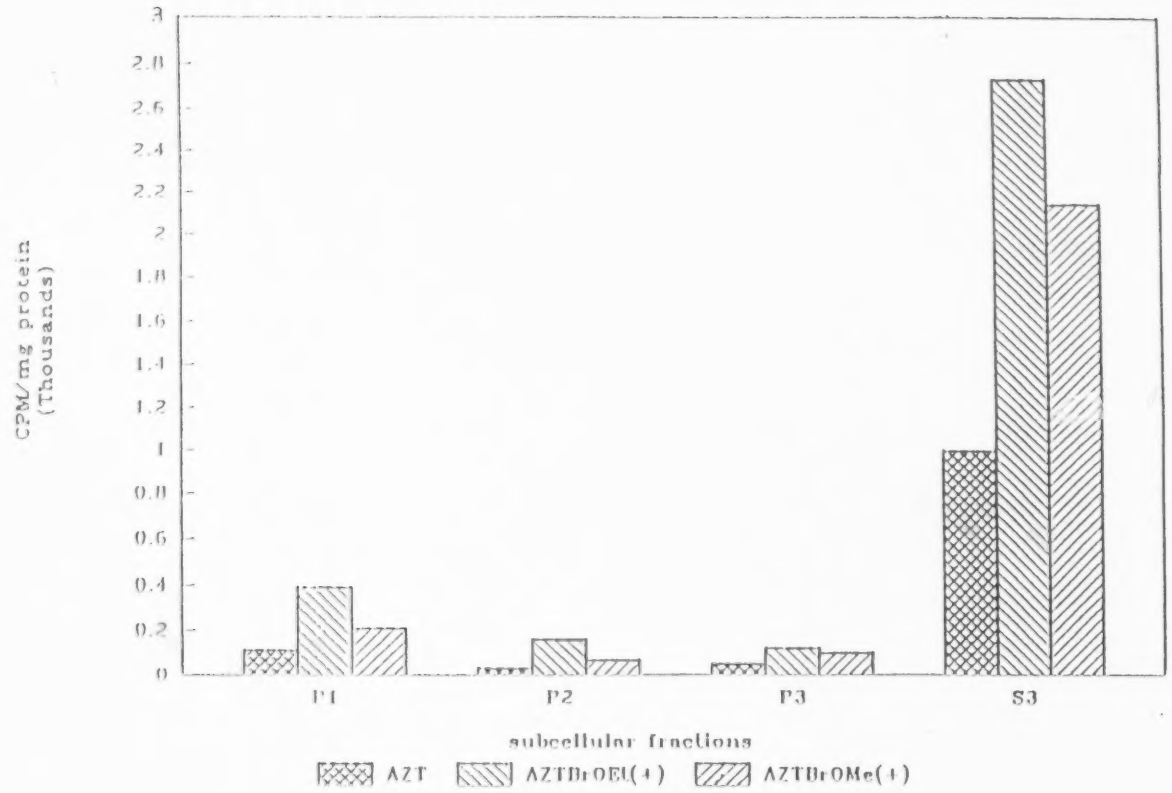


Figure 4.10.2. Distribution of [2-^{14}C]-AZT and [2-^{14}C]-trans-(+)-(5R,6R)-5-bromo-6-methoxy (or ethoxy)-5,6-dihydro analogs of AZT in subcellular fractions of mouse brain (2 μCi, specific activity 56 mCi/mmole; pooled brain samples from 5 mice).

4.11. Regional distribution of [2-^{14}C]-AZT and its [2-^{14}C]-5-bromo-6-methoxy (or ethoxy)-5,6-dihydro analogs in brain.

Although the precise cause of the HIV-related CNS disorder is unknown, histological studies indicate that loosely organized cellular infiltrates including macrophages, microglia, astrocytes, and scattered multinucleated cells are present in the AIDS dementia complex.[269,270] These cellular infiltrates are commonly found in basal ganglia, brain stem, and subcortical white matter, with the extent of infiltration correlating with the degree of dementia. Although the mode of HIV entry into the CNS remains unclear, HIV infection of the brain is suggested by the presence of HIV-1 antigen within the cerebrospinal fluid (CSF) and by intra-BBB synthesis of HIV-1-specific antibodies.[270] As reported in the literature[92], AZT distributes into CSF with CSF/plasma concentration ratios of 0.24-0.85. However, rat studies showed that less than 1.0% of injected AZT[134,135] penetrated into brain. It has been observed that [2-^{14}C]-5-bromo-6-methoxy (or ethoxy)-5,6-dihydro analogs of AZT resulted in higher concentrations of radioactivity in brain than for [2-^{14}C]-AZT, but that no differences in subcellular localization of radioactivity occurred within the brain. We then conducted a study of the regional distribution of [2-^{14}C]-AZT and [2-^{14}C]-trans-(+)-(5R,6R)-5-bromo-6-methoxy (or ethoxy)-5,6-dihydro analogs of AZT in mouse brain. The mouse brains collected 10 min after jugular vein injection were dissected into cerebellum, pons and medulla, hypothalamus, hippocampus, striatum, cortex and the rest of brain. Since the weight of each individual part was small and associated radioactivity was low, the individual parts from four (for AZT) and two (for 5,6-dihydro analogs) mice were pooled. They were weighed wet and analyzed by the standard combustion/liquid scintillation counting method described in Section 3.2.8.3 (page 73). The results calculated as CPM/mg regional tissue are shown in Table 4.11.1. The results obtained indicate that radioactivity resulting from all three compounds is relatively uniformly distributed into all regions of brain examined

with the exception of the hypothalamus where a higher concentration (about 2-fold compared to other regions) was observed. of [2-^{14}C]-trans-(+)-(5R,6R)-AZTBrOEt, levels of radioactivity in the hippocampus were also high, similar to those in the hypothalamus. The mechanism by which the HIV virus enters the brain, and whether the virus is localized preferentially in one part of the brain are not well understood. The results obtained in this study may serve as the basis for future investigation.

Table 4.11.1. Regional study of [2-^{14}C]-AZT and [2-^{14}C]-5-bromo-6-methoxy (or ethoxy)-5,6-dihydro analogs of AZT in mouse brain (2 μCi, specific activity 56 mCi/mmole).

	Radioactivity (CPM/mg) from pooled samples		
	[2-^{14}C]-AZT[a]	**[2-^{14}C]-trans-(+)-(5R,6R)-AZTBrOMe[b]**	**[2-^{14}C]-trans-(+)-(5R,6R)-AZTBrOEt[b]**
cerebellum	13.4	71.3	64.9
pons & medulla	14.8	65.4	60.6
hypothalamus	20.5	130.3	108.0
hippocampus	14.2	70.1	107.9
striatum	14.2	86.3	83.4
cortex	12.1	69.8	65.2
rest of brain	12.9	69.6	81.6

[a] Sample was pooled from four individual mice.
[b] Sample was pooled from two individual mice.

5. Summary and Conclusions

The development of anti-HIV therapy has resulted in the introduction of four nucleoside drugs for clinical use, i.e. AZT, ddI, ddC and d4T. These drugs all function to inhibit HIV RT, which plays a key role in replication of the virus. Recognition of toxicity, fast blood clearance and limited brain uptake, as well as the appearance of resistance, suggests that more potent agents with less toxicity, increased brain delivery, and improved pharmacokinetic profiles are needed. Current research underway includes the development of new agents with different mechanisms of action as well as improvement of the drugs already shown to be active. With regard to improving the therapeutic efficacy of current drugs, research to develop prodrugs of AZT and other anti-HIV nucleoside analogs has been very active. 5-Halo-6-alkoxy (or azido)-5,6-dihydro analogs of AZT, FLT and d4T have been synthesized as new anti-HIV agents and novel prodrugs for AZT, FLT and d4T.[313-315] Anti-HIV test results suggested that their activity against the HIV-1 virus *in vitro* is dependent on the nature of substituents at both C^5 and C^6 positions.[313-315] We have studied these 5-halo-6-alkoxy (or azido)-5,6-dihydro analogs of AZT, FLT and d4T as potential lipophilic prodrugs. From the investigations described in this thesis, the following summary and conclusions can be made:

1. The 5,6-dihydro analogs are equal or less potent anti-HIV agents than their corresponding parent compounds *in vitro*, depending upon the substituents at C^5 and C^6 of the 5,6-dihydro analogs. The 5-iodo (or bromo) analogs are generally more potent than their corresponding 5-chloro analogs. The 6-methoxy analogs are generally more active than the analogs with other alkoxy groups at the C^6 position. The stability study of 5-halo-6-methoxy-5,6-dihydro analogs of AZT in cell culture medium (MEMS/10%FBS) showed that the 5-iodo (or bromo)-6-methoxy-5,6-dihydro analogs of AZT gradually convert to AZT in the cell culture medium, whereas the 5-chloro-6-methoxy-5,6-dihydro

analog of AZT does not convert to AZT under these conditions. This indicates that the EC_{50} and IC_{50} values obtained for the 5-bromo (or iodo) analogs reflect the conversion from the 5,6-dihydro analogs to their corresponding parent compounds during *in vitro* incubation and that the EC_{50} and IC_{50} of the 5-chloro analogs are not due to a prodrug effect.

2. The 5,6-dihydro analogs of AZT, FLT and d4T are more lipophilic than their corresponding parent drugs. The determination of partition coefficients in the 1-octanol/water system indicated that 5-halo-6-methoxy-5,6-dihydro analogs of AZT are more lipophilic (P = 3.3-18.81) than AZT (P = 1.29). The 5-halo-6-methoxy-5,6-dihydro analogs of FLT and d4T are three to ten-fold and eight to twenty-eight fold more lipophilic than FLT (P = 0.5) and d4T (P = 0.12), respectively. Individual diastereomers possess different lipophilicities, suggesting that the configurations at C^5 and C^6 are determinants. The retention times of 5,6-dihydro analogs of AZT are significantly longer than that of AZT on reverse phase HPLC (10-24 min vs. 7.5 min) using methanol:water (6:4) at 1 mL/min, again indicating that the 5,6-dihydro analogs are more lipophilic than AZT. There is a linear correlation ($\log P = 0.061\ t_R - 0.2215$ [$R^2 = 0.90$]) between the partition coefficients (log P) and the retention times (t_R) for AZT and its 5-halo-6-methoxy-5,6-dihydro analogs. This may provide a tool for predicting partition coefficients of other compounds in this series (5-halo-6-alkoxy-5,6-dihydro analogs of AZT) from their retention times in reverse phase HPLC under the same conditions.

3. The 5,6-dihydro analogs, in most cases, have increased affinity than AZT for the mouse erythrocyte NBMPR-sensitive nucleoside transporter. Nucleosides are mainly transported across cell membranes by facilitated mechanisms. The NBMPR-sensitive nucleoside transporter (NT) is one of the better characterized transport systems, and accepts a wide range of substrates. Thymidine is known to traverse human erythrocyte membranes by this system. However, AZT and d4T as well as other ddNs have been shown to translocate through cell membranes by passive diffusion. FLT has been shown

to cross cell membrane by two mechanisms (NBMPR-sensitive and -insensitive). It is also known that thymidine transport inhibitors potentiate the efficacy of AZT.

The interaction of AZT and its 5,6-dihydro analogs with the NBMPR-sensitive NT has been investigated by determination of the inhibition constants (K_i) of AZT and its 5,6-dihydro analogs from thymidine influx competition experiments in a simple model (mouse erythrocytes). The K_i value represents the relative affinity of test compounds for the NBMPR-sensitive transport system and the results indicate that trans-5-halo-6-methoxy-5,6-dihydro analogs have higher affinity (K_i = 0.2-0.4 mM) for the NBMPR-sensitive NT system than does AZT (K_i = 1.3 mM). In contrast, cis-(+)-(5S,6R)-AZTClOMe (K_i >> 1.5 mM) has a decreased affinity relative to AZT. It appears that the affinity of these 5,6-dihydro analogs for the NBMPR-sensitive NT system is dependent on the configuration of substituents at the C^5 and C^6 positions. With increased affinity for the NBMPR-sensitive nucleoside transport system, these analogs may interfere with the cellular uptake of thymidine. However it is not known if this will result in significantly increased efficacy of AZT in a clinical setting.

4. The 5,6-dihydro analogs are not subject to phosphorolysis by *E. coli* thymidine phosphorylase, suggesting that the 5,6-dihydro analogs would be stable to phosphorolysis *in vivo*. Phosphorolysis of nucleoside analogs (with production of nucleobase and 1-phosphate ribose or 1-phosphate-2'-deoxy-ribose) is a catabolic reaction. Although AZT is stable toward thymidine phosphorolysis, d4T, DU and EDU underwent 10, 57 and 12% phosphorolysis. All 5-halo-6-methoxy (or azido)-5,6-dihydro analogs of AZT, d4T, DU and EDU examined were found to be stable toward phosphorolysis by *E. coli* thymidine phosphorylase.

5. *In vitro* conversion of the 5,6-dihydro analogs to AZT, FLT and d4T is dependent upon the nature of the halogen at the C^5 position. The conversion from 5,6-dihydro uracils to the parent uracils maybe a thiol-mediated process. Regeneration of AZT, FLT and d4T from their 5-halo-6-methoxy-5,6-dihydro analogs was investigated

following incubation of the individual 5,6-dihydro analog with glutathione, mouse whole blood or a mouse soluble liver enzyme fraction at 37°C. The results showed that both 5-iodo and 5-bromo-6-methoxy-5,6-dihydro analogs convert to their corresponding parent compounds to different degrees in the order of I > Br, whereas 5-chloro-6-methoxy-5,6-dihydro analogs were stable. All of the 5-halo-6-methoxy-5,6-dihydro analogs of AZT examined are stable upon incubation with phosphate buffer at 37°C for at least 24 h. A dependency of the % regeneration of the 5,6-double bond upon the nature of the halogen at the C^5 position has been demonstrated.

6. The 5-bromo (or iodo)-6-methoxy-5,6-dihydro analogs provided a considerable amount of AZT in mouse blood after i.v. injection. The prodrugs convert to AZT *in vivo*, and this conversion is dependent upon the nature of halogen at C^5 ($I \geq Br > Cl$). 5-Bromo (or iodo)-6-methoxy-5,6-dihydro analogs (144 μmol/kg) gave rise to AZT blood concentrations similar to those measured after equi-molar doses of AZT. On the other hand, the regeneration of AZT from its 5-chloro-6-methoxy-5,6-dihydro analog (240 μ mol/kg) is about 10 % of that for the bromo or iodo analogs at 15 min after administration of 144 μmol/kg, and AZT was not detectable after 90 min.

7. The 5,6-dihydro analogs enter the brain more readily than AZT. Increased lipophilicity should enable the 5,6-dihydro analogs to enter the brain more effectively. The biodistributions of ^{14}C labeled AZT and its 5-bromo-6-alkoxy (methoxy and ethoxy)-5,6-dihydro analogs were examined in mice after i.v. injection. Radioactivity recovered from brain for the [2-^{14}C]-5-bromo-6-methoxy or 6-ethoxy-5,6-dihydro analogs was approximately three times that after [2-^{14}C]-AZT was administered. The AUC for the [2-^{14}C]-5,6-dihydro analogs (53.8 and 51.5 % ID·min/g for AZTBrOMe and AZTBrOEt) in brain were significantly higher than that for [2-^{14}C]-AZT (17.4 % ID·min/g).

The radioactivity present in blood after dosing with the 5,6-dihydro analogs is significantly higher than that for AZT for a longer time period (after 2 h), suggesting that the 5,6-dihydro analogs undergo slower clearance or that this reflects a depot effect. In

addition to the elevated blood and brain levels of radioactivity, the radioactivity recovered from bone was similar after administration of equimolar doses of AZT and the 5,6-dihydro analogs, which may indicate an improved therapeutic index for the prodrug.

8. Examination of subcellular fractions of mouse brain after jugular vein injection of ^{14}C labeled AZT or its 5-bromo-6-methoxy (or ethoxy)-5,6-dihydro analogs, suggested that both AZT and the 5,6-dihydro analogs investigated had little specific binding to the particulate subcellular fractions P_1 (nuclei, cell debris), P_2 (mitochondria, synaptosome) and P_3 (microsomes). There was a considerable localization in the soluble fraction (S_3). Increased radioactivity was present in all the fractions after the prodrug administration relative to that after AZT administration.

9. Measurement of radioactivity in various regions of mouse brain after jugular vein injection of ^{14}C labeled AZT or its 5-bromo-6-methoxy (or ethoxy)-5,6-dihydro analogs indicated that radioactivity from both AZT and the prodrugs exhibited very little preferential regional localization in brain.

Overall, based on the the *in vitro* and *in vivo* evaluations of selected 5-halo-6-alkoxy (or azido)-5,6-dihydro analogs of AZT, FLT and d4T, it is clear that the 5,6-dihydro analogs represent a valuable approach to the design and development of prodrugs for nucleoside RT inhibitors.

Bibliography

1. **Gottlieb, M. S., Schroff, R., Schanker, H. M., Weisman, J. D., Fan, P. T., Wolf, R. A., and Saxon, A.** "*Pneumocystis* carinii pneumonia and mucosal candidiasis in previously healthy homosexual men. Evidence of a new acquired cellular immunodeficiency" N. Engl. J. Med. 305:1425-1431 (1981).

2. **Masur, H., Michelis, M. A., Greene, J. B., Onorato, I., Stouwe, R. A. V., Holzman, R. S., Wormser, G., Brettman, L., Lange, M., Murray, H. W., and Cunningham-Rundles, S.** "An outbreak of community-acquired pneumocystis carinii pneumonia. Initial manifestation of cellular immune dysfunction" N. Engl. J. Med. 305:1431-1438 (1981).

3. **Siegal, F. P., Lopez, C., Hammer, G. S., Brown, A. E., Kornfeld, S. J., Gold, J., Hassett, J., Hirschman, S. Z., Cunningham-Rundles, C., Adelsberg, B. R., Parham, D. M., Siegal, M., Cunningham-Rundles, S., and Armstrong, D.** "Severe acquired immunodeficiency in male homosexuals, manifested by chronic perianal ulcerative herpes simplex lesions" N. Engl. J. Med. 305:1439-1444 (1981).

4. **Bowen, D. L., Lane, H. L., and Fauci, A. S.** "Immunopathogenesis of the aquired immunodeficiency syndrome" Ann. Intern. Med. 103:704-709 (1985).

5. **Fauci, A. S., Masur, H., Gelmann, E. P., Markham, P. D., Hahn, B. H., and Lane, H. C.** "The aquired immunodeficiency syndrome: an update." Ann. Intern. Med. 102:800-813 (1985).

6. **Fauci, A. S.** "Immunologic abnormalities in the acquired immunodeficiency syndrome (AIDS)" Clin. Res. 32:491-499 (1985).

7. **Fauci, A. S.** "The human immunodeficiency virus: infectivity and mechanisms of pathogenesis" Science 239:617-622 (1988).

8. **Britton, C. B., and Miller, J. R.** "Neurologic complications in acquired immunodeficiency syndrome (AIDS)" Neurol. Clin. 2:315-339 (1984).

9. **Jordan, B. D., Navia, B. A., Petito, C., Cho, E.-S., and Price, R. W.** "Neurological syndromes complicating AIDS" Front. Radiat. Ther. Oncol. 19:82-87 (1985).

10. **Levy, R. M., Bredesen, D. E., and Rosenblum, M. L.** "Neurological manifestations of the acquired immunodeficiency syndrome (AIDS): Experience at UCSF and review of the literature" J. Neurosurg. 62:475-495 (1985).

11. **Navia, B. A., Jordan, B. D., and Price, R. W.** "The AIDS dementia complex: I. Clinical features" Ann. Neurol. 19:517-524 (1986).

12. **Price, R. W., Brew, B., Sidetis, J., Rosenblum, M., Scheck, A. C., and Cleary, P.** "The brain in AIDS: central nervous system HIV-1 infection and AIDS dementia complex" Science 239:586-592 (1988).

13. **McArthur, J. C.** "Neurologic manifestations of AIDS" Medicine 66:407-437 (1987).

14. **Centers for disease control.** "Human T-lymphotropic virus type III" MMWR. 35:542-549 (1986).

15. **Schinazi, R. F., Mead, J. R., and Feorino, P. M.** "Insights into HIV chemotherapy" AIDS Res. Hum. Retroviruses 8:963-1000 (1992).

16. The HIV/AIDS pandemic: 1993 overview (document WHO/GPA/CNP/EVA/93.1, Global program on AIDS, Geneva, 1993.

17. **Anderson, R. M., and May, R. M.** "AIDS: Epidemiology. Epidemiological parameters of HIV transmission" Nature 333:514-519 (1988).

18. **Curran, J. W., Lawrence, D. N., Jaffe, H., Kaplan, J. E., Zyla, L. D., Chamberland, M., Weinstein, R., Lui, K.-J., Schonberger, L. B., Spira, T. J., Alexander, W. J., Swinger,G., Ammann, A., Solomon, S., Auerbach, D., Mildvan, D., Stoneburner, R., Jason, J. M., Haverkos, H. W., and Evatt, B. L .** "Acquired immunodeficiency syndrome (AIDS) associated with transfusions" N. Engl. J. Med. 310:69-75 (1984).

19. **Fauci, A. S., Macher, A. M., Longo, D. L., Lane, H. C., Rook, A. H., Masur, H., and Gelmann, E. P.** "Acquired immunodeficiency syndrome: Epidemiologic, clinical, immunologic, and therapeutic considerations" Ann. Intern. Med. 100:92-96 (1984).

20. **Pizzo, P. A.** "Pediatric AIDS: Problems within problems" J. Infect. Dis. 161:316-325 (1990).

21. **Ryder, R. W., Nsa, W., Hassig, S. E., Behets, F., Rayfield, M., Ekungola, B., Nelson, A. M., Mulenda, U., Francis, H., Mwandagalirwa, K., Davachi, F., Rogers, M., Nzilambi, N., Greenberg, A., Mann, J., Quinn, T. C., Piot, P., and Curran, J. W.** "Perinatal transmission of the human immunodeficiency virus type 1 to infants of seropositive woman in Zaire" N. Engl. J. Med. 320:1637-1642 (1989).

22. **Fallon, J., Eddy, J., Weiner, L., and Pizzo, P. A.** "Human immunodeficiency virus infection in children" J. Pediatr. 114:1-30 (1989).

23. **Barré-Sinoussi, F., Chermann, J. C., Rey, F., Nugeyre, M. T., Chamaret, S., Gruest, J., Dauguet, C., Axler-Blin, C., Vézinet-Brun, F., Rouzioux, C., Rozenbaum, W., and Montagnier, L.** "Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS)" Science 220: 868-871 (1983).

24. **Gallo, R. C., Salahuddin, S. Z., Popovic, M., Shearer, G. M., Kaplan, M., Haynes, B. F., Palker, T. J., Redfield, R., Oleske, J., Safai, B., White, G., Foster, P., and Markham, P. D.** "Frequent detection and isolation of cytopathic retroviruses (HTLV-III) from patients with AIDS and at risk for AIDS" Science 224:500-503 (1984).

25. **Levy, J. A., Hoffman, A. D., Kramer, S. M., Landis, J. A., Shimabukuro, J. M., and Oshiro, L. S.** "Isolation of lymphocytopathic retroviruses from San Francisco patients with AIDS" Science 225:840-842 (1984).

26. **Gallo, R. C., Kalyanaraman, V. S., Sarngadharan, M. G., Sliski, A., Vonderheid, E. C., Maeda, M., Nakao, Y., Yamada, K., Ito, Y., Gutensohn, N., Murphy, S., Bunn, P. A. Jr., Catousky, D., Greaves, M. F., Blayney, D. W., Blattner, W., Jarrett, W. F. H., Hausen, H. Z., Seligmann, M., Brouet, J. C., Haynes, B. F., Jegasothy, B. V., Jaffe, E., Cossman, J., Broder, S., Fisher, R. I., Golde, D. W., and Robert-Guroff, M.** "Association of the human type C retrovirus with a subset of adult T-cell cancers" Cancer Res. 43:3892-3899 (1983).

27. **Popovic, M., Sarngadharan, M. G., Read, E., and Gallo, R. C.** "Detection, isolation, and continuous production of cytopathic retroviruses (HTLV-III) from patients with AIDS and pre-AIDS" Science 224:497-500 (1984).

28. **Nathanson, N., Georgsson, G., Pálsson, P. A., Najjar, J. A., Lutley, R., and Pétursson, G.** "Experimental visna in Icelandic sheep: The prototype lentiviral infection" Rev. Infect. Dis. 7:75-82 (1985).

29. **Cheevers, W. P., and McGuire, T. C.** "Equine infectious anemia virus: Innunopathogenesis and persistence" Rev. Infect. Dis. 7:83-88 (1985).

30. **Pedersen, N. C., Ho, E. W., Brown, M. L., and Yamamoto, J. K.** "Isolation of a T-lymphotropic virus from domestic cats with an immunodeficiency-like syndrome" Science 235:790-793 (1987).

31. **Coffin, J., Hasse, A., Levy, J. A., Montagnier, L., Oroszlan, S., Teich, N., Temin, H., Toyoshima, K., Varmus, H., Vogt, P., and Weiss, R.** "Human immunodeficiency viruses" Science 232:697 (1986).

32. **Gallo, R. C. and Montaigner, L.** "AIDS in 1988" Sci. Am. 259:41-48 (1988).

33. **Clavel, F., Guetard, D., Brun-Vezinet, F., Chamaret, S., Rey, M.-A., Santos-Ferreira, M. O., Laurent, A. G., Oauguet, C., Katlama, C., Rouzioux, C., Klatzman, D., Champalimaud, J. L., and Montagnier, L.** "Isolation of a new human retrovirus from West African patients with AIDS" Science 233:343-346 (1986).

34. **Gallo, R., Wong-Staal, F., Montagnier, L., Haseltine, W. A., and Yoshida, M.** "HIV/HTLV gene nomenclature (letter)" Nature (London) 333:504 (1988).

35. **Leis, J., Baltimore, D., Bishop, J. M., Coffin, J., Fleissner, A., Goff, S. P., Oroszlan, S., Robinson, H., Skalka, A. M., Temin, H. M., and Vogt, V.** "Standardized and simplified nomenclature for proteins common to all retroviruses" J. Virol. 62:1808-1809 (1988).

36. **Stevenson, M., Bukrinsky, M., and Haggerty, S.** "HIV-1 replication and potential targets for intervention" AIDS Res. Hum. Retroviruses 8:107-117 (1992).

37. **Klatzmann, D., Champagne, E., Chamaret, S., Gruest, J., Guetard, D., Hercend, T., Gluckman, J-C., and Montagnier, L.** "T-lymphocyte T4 molecule behaves as the receptor for human retrovirus LAV" Nature 312:767-768 (1984).

38. **Dalgleish, A. G., Beverley, P. C. L., Clapham, P. R., Crawford, D. H., Greaves, M. F., and Weiss, R. A.** "The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus" Nature 312:763-767(1984).

39. **Harper, M. E., Marselle, L. M., Gallo, R. C., and Wong-Staal, F.** "Detection of lymphocytes expressing human T-lymphotropic virus type III in lymph nodes and peripheral blood from infected individuals by *in situ* hybridization" Proc. Natl. Acad. Sci. U. S. A. 83:772-776 (1986).

40. **Watkins, B. A., Dorn, H. H., Kelly, W. B., Armstrong, R. C., Potts, B. J., Michaels, F., Kufta, C. V., and Dubois-Dalcq, M.** "Specific tropism of HIV-1 for microglial cells in primary human brain cultures" Science 249:549 (1990).

41. **Gartner, S., Markovits, P., Markovits, D. M., Betts, R. F., and Popovic, M.** "Virus isolation from and identification of HTLV-III/LAV-producing cells in brain tissue from a patient with AIDS" J. Am. Med. Assoc. 256:2365-2371 (1986).

42. **DeVita, V. T. Jr., Hellman, S., and Rosenberg, S. A.** "AIDS, etiology, diagnosis, treatment and prevention" 3rd edition, by J. B. Lippincott Company, Philadelphia, 1992.

43. **Levy, J. A.** "Pathogenesis of human immunodeficiency virus infection" Microbiol. Rev. 57:183-289 (1993).

44. **Wong-Staal, F., and Gallo, R. C.** "Human T-lymphotropic viruses" Nature 317:395-403 (1985).

45. **Gougeon, M.-L., and Montagnier, L.** "Apoptosis in AIDS" Science 260:1269-1270 (1993).

46. **Weiss, R. A.** "How does HIV cause AIDS" Science 160:1273-1279 (1993).

47. **Zagury, D., Bernard, J., Leonard, R., Cheynier, R., Feldman, M., Sarin, P. S., and Gallo, R. C.** "Long-term cultures of HTLV-III-infected T cells: A model of cytopathology of T-cell depletion in AIDS" Science 231:850-853 (1986).

48. **Yarchoan, R., Pluda, J. M., Perno, C-F., Mitsuya, H., and Broder, S.** "Anti-retroviral therapy of human immunodeficiency virus infection: Current strategies and challenges for the future" Blood 78(4):859-884 (1991).

49. **Mitsuya, H., Yarchoan, R., and Broder, S.** "Molecular targets for AIDS therapy" Science 249:1533-1544 (1990).

50. **Devita, V. T. Jr., Broder, S., Fauci, A. S., Kovacs, J. A., and Chabner, B. A.** "Developmental therapeutics and the acquired immunodeficiency Syndrome" Ann. Intern. Med. 106(4):568-581 (1987).

51. **De Clercq, E.** "HIV inhibitors targeted at the reverse transcriptase" AIDS Res. and Human Retroviruses 8:119-134 (1992).

52. **De Clercq, E.** "Potential drugs for the treatment of AIDS" J. Antimicrob. Chemother. 23, suppl. A. :35-46 (1989).

53. **Sandström, E., and Öberg, B.** "Antiviral therapy in human immunodeficiency virus infections" Drugs 45:488-508; 45:637-653 (1993).

54. **Smith, D. H., Byrn, R. A., Marsters, S. A., Gregory, T., Groopman, J. E., and Capon, D. J.** "Blocking of HIV-1 infectivity by a soluble, secreted form of the CD4 antigen" Science 238:1704-1707 (1987).

55. **Fisher, R. A., Bertonis, J. M., Meier, W, M. Johnson, V. A., Costopoulos, D. S., Liu, T., Tizard, R., Walker, B. D., Hirsch, M. S., Schooley, R. T., and Flavell, R. A.** "HIV infection is blocked *in vitro* by recombinant soluble CD4" Nature 331:76-78 (1988).

56. **Hussuy, R. E., Richardson, N. E., Kowalski, M., Brown, N. R., Chang, H-C., Siliciano, R. F., Dorfman, T., Walker, B., Sodroski, J., and Reinherz, E. L.** "A soluble CD4 protein selectively inhibits HIV replication and syncytium formation" Nature 331:78-81 (1988).

57. **Deen, K. C., McDougal, J. S., Inacker, R., Folena-Wasserman, G., Arthos, J., Rosenberg, J., Maddon, P. J., Axel, R., and Sweet, R. W.** "A soluble form of CD4 (T4) protein inhibitors AIDS virus infection" Nature 331:82-84 (1988).

58. **Traunecker, A., Lüke, W., and Karjalainen, K.** "Soluble CD4 molecules neutralize human immunodeficiency virus type 1" Nature 331:84-86 (1988).

59. **Kahn, J. O., Allan, J. D., Hodges, T. L., Kaplan, L. D., Arri, C. J., Fitch, H.F., Izu, A. E., Mordenti, J., Sherwin, S. A., Groopman, J. E., and Volberding, P. A.** "The safety and pharmacokinetics of recombinant soluble CD4 (*r*CD4) in subjects with the acquired immunodeficiency syndrome (AIDS) and complex" Ann. Intern. Med. 112:254-261 (1990).

60. **Schooley, R. T., Merigan, T. C., Gaut, P., Hirsch, M. S., Holodniy, M., Flynn, T., Liu, S., Byington, R. E., Henochowicz, S., Gubish, E., Spriggs, D., Kufe, D., Schindler, J., Dawson, A., Thomas, D., Hanson, D. G., Letwin, B., Liu, T., Gulinello, J., Kennedy, S., Fisher, R., and Ho, D. D.** "Recombinant soluble CD4 therapy in patients with the acquired immunodeficiency syndrome (AIDS) and AIDS-related complex" Ann. Intern. Med. 112:247-253 (1990).

61. **Ueno, R., and Kuno, S.** "Dextran sulfate, a potent anti-HIV agent *in vitro* having synergism with zidovudine" Lancet 1:1379 (1987).

62. **Ito, M., Baba, M., Sato, A., Pauwels, R., De Clercq, E., and Shigeta,S.** "Inhibitory effect of dextran sulfate and heparin on the replication of human immunodeficiency virus (HIV) *in vitro*" Antiviral Res. 7:361-367 (1987).

63. **Nakashima, H., Kido, Y., Kobayashi, N., Motoki, Y., Neushul, M., and Yamamoto, N.** "Purification and characterization of an avian myeloblastosis and human immunodeficiency virus reverse transcriptase inhibitor, sulfated polysaccharides extracted from sea algae" Antimicrob. Agents Chemother. 31:1524-1528 (1987).

64. **Mitsuya, H., Looney, D. J., Kuno, S., Ueno, R., Wong-Staal, F., and Broder, S.** "Dextran sulfate suppression of viruses in the HIV family: inhibition of virion binding to $CD4^+$ cells" Science 240:646-649 (1988).

65. **Baba, M., Pauwels, R., Balzarini, J., Arnout, J., Desmyter, J., and De Clercq, E.** "Mechanism of inhibitory effect of dextran sulfate and heparin on replication of human immunodeficiency virus *in vitro*" Proc. Natl. Acad. Sci. U. S. A.. 85:6132-6136 (1988).

66. **Abrams, D. I., Kuno, S., Wong, R., Jeffords, K., Nash, M., Molagham, J. B., Gorter, R., and Ueno, R.** "Oral dextran sulfate (UA 001) in the treatment of the acquired immunodeficiency syndrome (AIDS) and AIDS-related complex" Ann. Intern. Med. 110:183-188 (1989).

67. **Lorentsen, K., Hendrix, C., Collins, J., Eckel, R., Petty, B., and Lietman, P.** "Dextran sulfate is poorly absorbed after oral administration" Ann. Intern. Med. 111:561-566 (1989).

68. **Hartman, N. R., Johns, D. G., and Mitsuya, H.** "Pharmacokinetic analysis of dextran sulfate in rats as pertaining to its clinical usefulness for therapy of HIV infection" AIDS Res. Human Retroviruses 6:805-812 (1990).

69. **Weiss, R. A., Clapham, P. R., Cheingsong-Popov, R., Dalgleish, A. G., Carne, C. A., Weller, I. V. D., and Tedder, R. S.** "Neutralization of human T-lymphotropic virus type III by sera of AIDS and AIDS-risk patients" Nature 316:69-72 (1985).

70. **Robert-Guroff, M., Brown, M., and Gallo, R. C.** "HTLV-III-neutralizing antibodies in patients with AIDS and AIDS-related complex" Nature 316:72-74 (1985).

71. **Lavie, G., Valentine, F., Levin, B., Mazur, Y., Gallo, G., Lavie, D., Weiner, D., and Meruelo, D.** "Studies of the mechanisms of action of the antiretroviral agents hypericin and pseudohypericin" Proc. Natl. Acad. Sci. U. S. A.. 86:5963-5967 (1989).

72. **Yarchoan, R., Mitsuya, H., Myers, CE., and Broder, S.** "Clinical pharmacology of 3'-azido-2',3'-dideoxythymidine (zidovudine) and related dideoxynucleosides" N. Engl. J. Med. 321:726-738 (1989).

73. **Mitsuya, H., Weinhold, K. J., Furman, P. A., Clair, M. H., Lehrman, S. N., Gallo, R. C. Bolognes, D., Barry, D. W., and Broder, S.** "3'-Azido-3'-deoxythymidine (BW A509U): an antiviral agent that inhibits the infectivity and cytopathic effect of human T-lynphotropic virus type III/lymphadenopathy-associated virus in vitro" Proc. Natl. Acad. Sci. U. S. A. 82:7096-7100 (1985).

74. **Yarchoan, R., Weinhold, K. J., Lyerly, H. K., Gelmann, E., Blum, R. M., Shearer, G. M., Mitsuya, H., Collins, J. M., Myers, C. E., Klecker, R. W., Markham, P. D., Durack, D. T., Lehrman, S. N., Barry, D. W., Fischl, M. A., Gallo, R. C., Bolognesi, D. P., and Broder, S.** "Administration of 3'-azido-3'-deoxythymidine, an inhibitor of HTLV III/LAV replication, to patients with AIDS-related complex." Lancet 1:575-580 (1986).

75. **Mitsuya, H., and Broder, S.** "Inhibition of the *in vitro* infectivity and cytopathic effect of human T-lymphotropic virus type-III/lymphadenopathy virus-associated virus (HTLV-III/LAV) by 2',3'-dideoxynucleosides" Proc. Natl. Acad. Sci. U. S. A. 83:1911-1915 (1986).

76. **Buenger, G. S.** "Synthesis of new dideoxynucleosides of antiviral interest" Diss. Abstr. Int. [B] 51:5869 (1991).

77. **Palomino, E., Meltsner, B. R., Kessel, D., and Horwitz, J. P.** "Synthesis and *in vitro* evaluation of some modified 4-thiopyrimidine nucleosides for prevention or reversal of AIDS-associated neurological disorders" J. Med. Chem. 33:258-263 (1990).

78. **McQuade, T. J., Tomasselli, A. G., Liu, L., Karacostas, V., Moss, B., Sawyer, T. K., Heinrikson, R. L., and Tarpley, W. G.** "A synthetic HIV-1 protease inhibitor with antiviral activity arrests HIV-like particle maturation" Science 247:454-456 (1990).

79. **Huff, J. R.** "HIV protease: A novel chemotherapeutic target for AIDS" J. Med.Chem. 34:2306-2314 (1991).

80. **Wlodawer, A., and Erickson, J. W.** "Structure-based inhibitors of HIV-1 protease" Ann. Rev. Biochem. 62:543-585 (1993).

81. **Craig, J. C., Duncan, I. B., Hockley, D., Grief, C., Roberts, N. A., and Mills, J. S.** "Antiviral properties of Ro 31-8959, an inhibitor of human immunodeficiency virus (HIV) proteinase" Antiviral Res. 16:295-305 (1991).

82. **Jacobsen, H., Craig, C. J., Duncan, I. B., Krohn, A., Mous, J., and Yasargil, K.** "Cell culture-selection and characterization of variant HIV-1 with reduced sensitivity to an inhibitor of the viral protease" J. Cell Biochem. Suppl. 17E:90 (1993).

83. **Johnston, M. I., and Hoth, D. F.** "Present status and future prospects for HIV therapies" Science 260:1286-1293 (1993).

84. **Lam, P. Y. S., Jadhav, P. K., Eyermann, C. J., Hodge, C. N., Ru, Y., Bacheler, L. T., Meek, J. L., Chang, C.-H., Weber, P. C., Jackson, D. A., Sharpe, T. R., and Erickson-Viitanen, S.** "Rational design of potent, bioavailable, nonpeptide cyclic ureas as HIV protease inhibitors" Science 263:380-384 (1994).

85. **Horwitz, J. P., Chua, J. and Noel, M.** "Nucleosides: V. The monomesylates of 1-(2'-deoxy-beta-D-lyxofuranosyl)thymidine" J. Org. Chem. 29:2076-2078 (1964).

86. **Fischl, M. A., Richman, D. D., Grieco, M. H., Gottlieb, M. S., Volberding, P. A., Laskin, O. L., Leedom, J. M., Groopman, J. E., Mildvan, D., Schooley, R. T., Jackson, G. G., Durack, D. T., King, D. and the AZT collaborative working group.** "The efficacy of azidothymidine (AZT) in the treatment of patients with AIDS and AIDS-related complex" N. Engl. J. Med. 317:185-191 (1987).

87. **Yarchoan, R., Berg, G., Brouwers, P., Fischl, M. A., Spitzer, A. R., Wichman, A., Grafman, J., Thomas, R. V., Safai, B., Brunetti, A., Perno, C. F., Schmidt, P. J., Larson, S. M., Myers, C. E., and Broder, S.** "Response of human-immunodeficiency-virus-associated neurological disease to 3'-azido-3'-deoxythymidine" Lancet 1:132-135 (1987).

88. **Schmitt, F. A., Bigley, J. W., Mckinnis, R., Logue, P. E., Evans, R. W., Drucker, J. L. and the AZT collaborative working group.** "Neuropsychological outcome of zidovudine (AZT) treatment of parients with AIDS and AIDS-related complex" N. Engl. J. Med. 319:1573-1578 (1988).

89. **Pizzo, P. A., Eddy, J., Falloon, J., Balis, F. M., Murphy, R. F., Moss, H., Wolters, P., Brouwers, P., Jarosinski, P., Rubin, M., Broder, S., Yarchoan, R., Brunetti, A., Maha, M., Nusinoff-Lehrman, S., and Poplack, D. G.** "Effect of continuous intravenous infusion zidovudine (AZT) in children with sympotomatic HIV infection" N. Engl. J. Med. 319:889-896 (1988).

90. **Kolata, G.** "Imminent marketing of AZT raises problems" Science 235:1462-1463 (1987).

91. **Fischl, M. A., Richman, D. D., Hansen, N., Collier, A. C., Carey, J. T., Para, M. F., Hardy, W. D., Dolin, R., Powderly, W. G., Allan, J. D., Wong, B., Merigan, T. C., McAuliffe, V. J., Hyslop, N. E., Rhame, F. S., Baifour, H. H., Spector, S. A., Volberding, P., Pettinelli, C., Anderson, J., and the AIDS clinical trials group.** "The safety and efficacy of zidovudine (AZT) in the treatment of subjects with mildly symptomatic human immunodeficiency virus type 1 (HIV) infection" Ann. Intern. Med. 112:727-737 (1990).

92. **Volberding, P. A., Lagakos, S. W., Koch, M. A., Pettinelli, C., Myers, M. W., Booth, D. K., Balfour, H. H., Reichman, R. C., Bartlett, J. A., Hirsch, M. S., Murphy, R. L., Hardy, W. D., Soeiro, R., Fischl, M. A., Bartlett, J. G., Merigan, T. C., Hyslop, N. E., Richman, D. D., Valentine, F. T., Corey, L., and the AIDS clinical trials group of the National Institute of Allergy and Infectious Diseases.** "Zidovudine in asymptomatic human immunodeficiency virus infection" N. Engl. J. Med. 322:941-949 (1990).

93. **Furman, P. A., Fyfe, J. A., St. Clair, M. H., Weinhold, K., Rideout, J. L., Freeman, G. A., Lehrman, S. N., Bolognesi, D. P., Broder, S., Mitsuya, H., and Barry, D. W.** "Phosphorylation of 3'-azido-3'-deoxythymidine and selective interaction of the 5'-triphosphate with human immunodificiency virus reverse transcriptase." Proc. Natl. Acad. Sci. U. S. A.. 83:8333-8337 (1986).

94. **Balzarini, J., Herdewijn., P., and De Clercq, E.** "Differential patterns of intracellular metabolism of 2',3'-didehydro-2',3'-dideoxythymidine and 3'-azido-2',3'-dideoxythymidine, two potent anti-human immunodeficiency virus compounds" J. Biol. Chem. 264:6127-6133 (1989).

95. **Perno, C. F., Yarchoan, R., Cooney, D. A., Hartman, N. R., Gartner, S., Popovic, M., Hao, Z., Gerrard, T. L., Wilson, Y. A., Johns, D. G., and Broder, S.** "Inhibition of human immunodeficiency virus (HIV-1/HTLV-III$_{Ba-L}$) replication in fresh and cultured human peripheral blood monocytes/macrophages by azidothymidine and related 2',3'-deoxynucleosides" J. Exp. Med. 168:1111-1125 (1988).

96. **Richman, D. D., Kornbluth, R. S., and Carson, D. A.** "Failure of dideoxynucleosides to inhibit human immunodeficiency virus replication in cultured human macrophages" J. Exp. Med. 166:1144-1149 (1987).

97. **Dahlberg, J. E., Mitsuya, H., Blam, S. B., Broder, S., and Aaronson, S. A.** "Broad spectrum antiretroviral activity of 2',3'-dideoxynucleosides" Proc. Natl. Acad. Sci. U. S. A. 84:2469-2473 (1987).

98. **Mitsuya, H., Jarrett, R. F., Matsukura, M., diMarzo Veronese, F., deVico, A. L., Sarngadharan, M. G., John, D. G., Reitz, M. S., and Broder, S.** "Long-term inhibition of human T-lymphadenopathy-associated virus (human immunodeficiency virus) DNA synthesis and RNA expression in T cells protected by 2',3'-dideoxynucleosides *in vitro*" Proc. Natl. Acad. Sci. U. S. A. 84:2033-2037 (1987).

99. **Yarchoan, R., and Broder, S.** "Development of antiretroviral therapy for the acquired immunodeficiency syndrome and related disorders" N. Engl. J. Med. 316:557-564 (1987).

100. **Larder, B. A., Darby, G., and Richman, D.D.** "HIV with reduced sensitivity to zidovudine (AZT) isolated during prolonged therapy" Science 243:1731-1734 (1989).

101. **Blum, M. R., Liao, H. T., Good, S. S. and De Miranda, P.** "Pharmacokinetics and bioavailability of zidovudine in humans" Am. J. Med. 85:189-194 (1988).

102. **Good, S. S., Koble, C. S., Crouch, R., Johnson, R. L., Rideout, J. L., and Miranda, P. De.** "Isolation and characterization of an ether glucuronide of zidovudine, a major metabolite in monkeys and humans" Drug Metab. Dispos. 18:321-326 (1990).

103. **Wilde, M. I., and Langtry, H. D.** "Zidovudine. An update of its pharmacodynamic and pharmacokinetic properties, and therapeutic efficacy" Drugs 46: 515-578 (1993).

104. **Eickhoff, T. C.** "The acquired immunodeficiency syndrome (AIDS) and infection with the human immunodeficiency virus (HIV)" Ann. Intern. Med. 108:460-469 (1988).

105. **De Miranda, P., Good, S. S., Blum, M. R., Thomas, R. V., Yarchoan, R., and Broder, S.** "The effect of probenecid on the pharmacokinetic disposition of azidothymidine (AZT)" Presented at the international conference on acquired immunodeficiency syndrome (AIDS), Paris, June 23-25 (1986).

106. **Good, S. S., Durack, D. T., and De Miranda, P.** "Biotransformation in various species and in humans of 3'-azido-3'-deoxythymidine, a potential agent for treatment of AIDS" Fed. Proc. 45:444 (1986).

107. **Clumeck, N.** "Current use of anti-HIV drugs in AIDS" J. Antimicrob. Chemother. 32 (suppl.A):133-138 (1993).

108. **Richman, D. D., Fischl, M. A., Grieco, M. H., Gottlieb, M. S., Volberding, P. A., Laskin, O. L., Leedom, J. M., Groopman, J. E., Mildvan, D., Hirsch, M. S., Jackson, G. G., Durack, D. T., Nusinoff-Lehrman, S., and the AZT collaborative working group**. "The toxicity of azidothymidine (AZT) in the treatment of patients with AIDS and AIDS-related complex" N. Engl. J. Med. 317:192-197 (1987).

109. **Dalakas, M. C., Illa, I., Pezeshkpour, G. H., Laukaitas, J. P., Cohen, B., and Griffin, J. L.** "Mitochondrial myopathy caused by long-term zidovudine therapy" N. Engl. J. Med. 322:1098-1105 (1990).

110. **Groopman, J. E.** "Zidovudine intolerance" Rev. Infect. Dis. 12:S500-S506 (1990).

111. **Cretton, E. M., Xie, M-Y, Bevan, R. J., Goudgaon, N. M., Schinazi, R. F., and Sommadossi, J-P.** "Catabolism of 3'-azido-3'-deoxythymidine in hepatocytes and liver microsomes, with evidence of formation of 3'-amino-3'-deoxythymidine, a highly toxic catabolite for human bone marrow cells" Mol. Pharmacol. 39:258-266 (1991).

112. **Lamperth, L., Dalakas, M. C., Dagani, F., Anderson, J., and Ferrari, R.** "Abnormal skeletal and cardiac muscle mitochondria induced by zidovudine (AZT) in human muscle in vitro and in an animal model" Lab. Invest. 65:742-751 (1991).

113. **Lewis, W., Gonzalez, B., Chomyn, A., and Papoian, T.** "Zidovudine induces molecular, biochemical, and ultrastructural changes in rat skeletal muscle mitochondria" J. Clin. Invest. 89:1354-1360 (1992).

114. **Lewis, W., Papoian, T., Gonzalez, B., Louie, H., Kelly, D. P., Payne, R. M., and Grody, W. W.** "Mitochondrial ultrastructural and molecular changes induced by zidovudine in rat hearts" Lab. Invest. 65:228-236 (1991).

115. **Chen, C. H., Vazquez-Padua, M., and Cheng, Y. C.** "Effect of anti-human immunodeficiency virus nucleoside analogs on mitochondrial DNA and its implication for delayed toxicity" Mol. Pharmacol. 39:625-628 (1991).

116. **Simpson, M. V., Chin, C. D., Keilbaugh, S. A., Lin, T. S., and Prusoff, W. H.** "Studies on the inhibition of mitochondrial DNA replication by 3'-azido-3'-deoxythymidine and other dideoxynucleoside analogs which inhibit HIV-1 replication" Biochem. Pharmacol. 38:1033-1036 (1989).

117. **Pezeshkpour, G., Krarup, C., Buchthal, F., Dimauro, S., Bresolin, N., and McBurney, J.** "Peripheral neuropathy in mitochondrial disease" J. Neurol. Sci. 77:285-304 (1987).

118. **Husson, R., and Pizzo, P. A.** "The use of nucleoside analogues in the treatment of HIV-infected children" AIDS Res. Hum. Retroviruses 8:1059-1064 (1992).

119. **Jackson, G. G., Paul, D. A., Falk, L. A., Rubenis, M., Despotes, J. C., Mack, D., Knigge, M., and Emeson, E. E.** "Human immunodeficiency virus (HIV) antigenemia (p24) in the acquired immunodeficiency syndrome (AIDS) and the effect of treatment with zidovudine (AZT)" Ann. Intern. Med. 108:175-180 (1988).

120. **Richman, D. D., Havlir, D., Corbeil, J., Looney, D., Ignacio, C., Spector, S. A., Sullivan, J., Cheeseman, S., Barringer, K., Pauletti, D., Shih, C.-K., Myers, M., and Griffin, J.** "Nevirapine resistance mutations of human

immunodeficiency virus type 1 selected during therapy" J. Virol. 68:1660-1666 (1994).

121. **Klecker, R. W. Jr., Collins, J. M., Yarchoan, R., Thomas, R., Jenkins, J. F., Broder, S., and Myers, C. E.** "Plasma and cerebrospinal fluid pharmacokinetics of 3'-azido-3'-deoxythymidine: A novel pyrimidine analog with potential application for the treatment of patients with AIDS and related diseases" Clin Pharmacol Ther 41:407-412 (1987).

122. **Stretcher, B. N., Pesce, A. J., Murray, J. A., Hurtubise, P. E., Vine, W. H., and Frame, P. T.** "Concentrations of phosphorylated zidovudine (ZDV) in patient leukocytes do not correlate with ZDV dose or plasma concentrations" Ther. Drug Monit. 13:325-331 (1991).

123. **Shaw, G. M., Harper, M. E., Hahn, B. H., Epstein, L. G., Gajdusek, D. C., Price, R. W., Navia, B. A., Petito, C. K., O'Hara, C. J., Groopman, J. E., Cho, E.-S., Oleske, J. M., Wong-Staal, F., and Gallo, R. C.** "HTLV-III infection in brains of children and adults with AIDS encephalopathy" Science 227:177-182 (1985).

124. **Ho, D. D., Rota, T. R., Schooley, R. T., Kaplan, J. C., Allan, J. D., Groopman, J. E., Resnick, L., Felsenstein, D., Andrews, C. A., and Hirsch, M. S.** "Isolation of HTLV-III from cerebrospinal fluid and neural tissues of patients with neurologic syndromes related to the acquired immunodeficiency syndrome" N. Engl. J. Med. 313:1493-1497 (1985).

125. **Resnick, L., diMarzo-Veronese, F., Schüpbach, J., Tourtellotte, W. W., Ho, D. D., Müller, F., Shapshak, P., Vogt, M., Groopman, J. E., Markham, P. D., and Gallo, R. C.** "Intra-blood-brain-barrier synthesis of HTLV-III-specific IgG in patients with neurologic symptoms associated with AIDS or AIDS-related complex" N. Engl. J. Med. 313:1498-1504 (1985).

126. **Michaels, J., Sharer, L. R., and Epstein, L. G.** "Human immunodeficiency virus type 1 (HIV-1) infection of the nervous system:a review" Immunodef. Rev. 1:71-104 (1988).

127. **Navia, B. A., Cho, E. S., Petito, C. K., and Price, R. W.** "The AIDS dementia complex. II. Neuropathology." Ann. Neurol. 19:525-535 (1986).

128. **Gallo, R., Rossi, A. D., Amadori, A., Tavolata, B., and Chieco-Bianchi, L.** "Central nervous system involvement in HIV infection" AIDS Res. Human Retroviruses 4:211-221 (1988).

129. **Pumarola-Sune, T., Navia, B. A., Cordon-Cardo, C., Cho, E. S., and Price, R. W.** "HIV antigen in the brains of patients with the AIDS dementia complex" Ann. Neurol. 21:490-496 (1987).

130. **Koenig, S., Gendelman, H. E., Orenstein, J. M., Dal Canto, M. C., Pezeshkpour, G. H., Yungbluth, M., Janotta, F., Aksamit, A., Martin, M., and Fauci, A. S.** "Detection of AIDS virus in macrophages in brain tissue from AIDS patients with encephalopathy" Science 233:1089-1093 (1986).

131. **Levy, J. A., Shimabukuro, J., Hollander, H., Shimabukuro, J., Mills, J., and Kaminsky, L.** "Isolation of AIDS associated retroviruses from cerebrospinal fluid and brain of patients with neurological symptoms" Lancet 2:586-588 (1985).

132. **Chiodi, F., Albert, J., Olausson, E., Norkrans, G., Hagberg, L., Sonnerborg, A., Asjo, B., and Fenyo, E.-M.** "Isolation frequency of human immunodeficiency virus from cerebrospinal fluid and blood of patients with varying severity of HIV infection" AIDS Res. Hum. Retroviruses 4:351-358 (1988).

133. **Gartner, S., Markovits, P., Markovitz, D. M., Japlan, M. H., Gallo, R. C., and Popovic, M.** "The role of mononuclear phagocytes in HTLV-III/LAV infection" Science 233:215-219 (1986).

134. **Ellison, S., Terasaki, T., and Pardridge, W. M.** "AZT and dideoxynucleosides do not cross the blood-brain barrier" Clin. Res. 36: 117A (1988).

135. **Terasaki, T., and Pardridge, W. M.** "Restricted transport of 3'-azido-3'-deoxythymidine and deoxynucleosides through the blood-brain barrier" J. Infect. Dis. 158:630-632 (1988).

136. **Cornford, E. M., and Oldendorf, W. H.** "Independent blood-brain barrier transport systems for nucleic acid precursor" Biochim. Biophys. Acta. 394:211-219 (1975).

137. **Spector, R., and Berlinger, W. G.** "Localization and mechanism of thymidine transport in the central nervous system" J. Neurochem. 39:837-841 (1982).

138. **Rooke, R., Tremblay, M., Soudeyns, H., DeStephano, L., Yao, X-J, Fanning, M., Montaner, J. S. G., O'Shaughnessy, M., Gelmon, K., Troukas, C., Gill, J., Ruedy, J., Wainberg, M. A., and the Canadian Zidovudine Multi-Centre Study Group.** "Isolation of drug-resistant variants of HIV-1 from patients on long-term zidovudine therapy" AIDS 3:411-415 (1989).

139. **Land, S., McPhee, D., Birch, C., Doherty, R., Cooper, D., and Gust, I.** "Decreased in vitro susceptibility to zidovudine of HIV isolates obtained from patients with AIDS" J. Infect Dis. 161:326-329 (1990).

140 **Richman, D. D., Grimes, J. M., and Lagakos, S. W.** "Effect of stage of disease and drug dose on zidovudine susceptibilities of isolates of human immunodeficiency virus" J. Acquir. Immune. Defic. Syndr. 3:743-746 (1990).

141. **Mayers, D. L., McCutchan, F. E., Sanders-Buell, E. E., Merritt, L. I., Dilworth, S., Fowler, A. K., Marks, C. A., Ruiz, N. M., Richman, D. D., Roberts, C. R., and Burke, D. S.** "Characterization of HIV isolates arising after prolonged zidovudine therapy" J. Acquir. Immune. Defic. Syndr. 5:749-759 (1992).

142. **Boucher, C. A. B., Tersmette, M., Lange, J. M. A., Kellam, P., deGoede, R. E. Y., Mulder, J. W., Darby, G., Goudsmit, J., and Larder, B. A.** "Zidovudine sensitivity of human immunodeficiency viruses from high-risk, symptom-free individuals during therapy" Lancet 336:585-590 (1990).

143. **Larder, B. A., and Kemp, S. D.** "Multiple mutations in HIV-1 reverse transcriptase confer high-level resistance to zidovudine (AZT)" Science 246:1155-1158 (1989).

144. **Kellam, P., Bouchen, C. A. B., and Larder, B. A.** "Fifth mutation in human immunodeficiency virus type 1 reverse transcriptase contributes to the development of high-level resistance to zidovudine" Proc. Natl. Acad. Sci. U. S. A. 89:1934-1938 (1992).

145. **Dornsife, R. E., St. Clair, M. H., Huang, A. T., Panella, T. J., Koszalka, G. W., Burns, C. L., and Averett, D. R.** "Anti-human immunodeficiency virus synergism by zidovudine (3'-azidothymidine) and didanosine (dideoxyinosine) contrasts with their additive inhibition of normal human marrow progenitor cells" Antimicrob. Agents Chemother. 35:322-328 (1991).

146. **Johnson, V. A., Merrill, D. P., Videler, J. A., Chou, T.-C., Byington, R. E., Eron, J. J., D'Aquila, R. T., and Hirsch, M. S.** "Two-drug combinations of zidovudine, didanosine, and recombinant interferon-α A inhibit replication of zidovudine-resistant human immunodeficiency virus type 1 synergistically in vitro" J. Infect. Dis. 164:646-655 (1991).

147. **Richman, D., Rosenthal, A. S., Skoog, M., Eckner, R. J., Chou, T.-C., Sabo, J. P., and Merluzzi, V. J.** "B1-RG-587 is active against zidovudine-resistant human immunodeficiency virus type 1 and synergistic with zidovudine" Antimicrob. Agents Chemother 35:305-308 (1991).

148. **Goldman, M. E., Nunberg, J. H., O'Brien, J. A., Quintero, J. C., Schleif, W. A., Freund, K. F., Gaul, S. L., Saari, W. S., Wai, J. S., Hoffman, J. M., Anderson, P. S., Hupe, D. J., Emini, E. A., and Stern, A. M.** "Pyridinone

derivatives: specific human immunodeficiency virus type 1 reverse transcriptase inhibitors with antiviral activity" Proc. Natl. Acad. Soc. U.S.A. 88:6863(1991).

149. **St. Clair, M. H., Martin, J. L., Tudor-William, G., Bach, M. C., Vavro, C. L., King, D. M., Kellam, P., Kemp, S. D., and Larder, B. A.** "Resistance to ddI and sensitivity to AZT induced by a mutation in HIV-1 reverse transcriptase" Science 253:1557-1559 (1991).

150. **Larder, B. A., Chesebro, B., and Richman, D. D.** "Susceptibilities of zidovudine-susceptible and -resistant human immunodeficiency virus isolates to antiviral agents determined by using a quantitative plaque reduction assay" Antimicrob. Agents Chemother. 34:436-441 (1990).

151. **Richman, D. D., Rosenthal, A. S., Skoog, M., Eckner, R. J., Chou, T.-C., Sabo, J. P., and Merluzzi, V. J.** "BI-RG-587 is active against zidovudine-resistant human immunodeficiency virus type 1 and synergistic with zidovudine" Antimicrob. Agents Chemother. 35:305-308 (1991).

152. **Dueweke, T. J., Pushkarskaya, T., Poppe, S. M., Swaney, S. M., Zhao, J. Q., Chen, I. S. Y., Stevenson, M., and Tarpley, W. G.** "A mutation in reverse transcriptase of bis(heteroaryl)piperazine-resistant human immunodeficiency virus type 1 that confers increased sensetivity to other nonnucleoside inhibitors" Proc. Natl. Acad. Soc. U. S. A. 90:4713-4717 (1993).

153. **Richman, D. D.** "Emergence of mutant HIV reverse transcriptase conferring resistance to AZT" J. Enzy. Inhib. 6:55-64 (1992).

154. **Langtry, H. D., and Campoli-Richards, D. M.** "Zidovudine. A review of its pharmacodynamics and pharmacokinetic properties, and therapeutic efficacy" Drugs 37:408-450 (1989).

155. **Faulds, D., and Brogden, R. N.** "Didanosine. A review of its antiviral activity, pharmacokinetic properties and therapeutic potential in human immunodeficiency virus infection" Drugs 44:94-116 (1992).

156. **Whittington, R., and Brogden, R. N.** "Zalcitabine. A review of its pharmacology and clinical potential in acquired immunodeficiency syndrome (AIDS)" Drugs 44:656-683 (1992).

157. "Fourth drug to fight HIV infection approved by FDA" Chem. Eng. News July 4, 1994.

158. **Browne, M. J., Mayer, K. H., Chafee, S. B. D., Dudley, M. N., Posner, M. R., Steinberg, S. M., Graham, K. K., Geletko, S. M., Zinner, S. H., Denman, S. L., Dunkle, L., Kaul, S., McLaren, C., Skowron, G., Kouttab, N. M.,**

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Kennedy, T. A., Weiberg, A. B., and Curt, G. H. "2',3'-Didehydro-3'-deoxythymidine (d4T) in patients with AIDS or AIDS-related complex: a phase I trial" J. Infect. Dis. 167:21-29 (1993).

159. **Nasr, M., Cradock, J., and Johnston, M. I.** "Computer-assisted structure-activity correlations of halodideoxynucleoside analogs as potential anti-HIV drugs" AIDS Res. Hum. Retroviruses 8:135-144 (1992).

160. **Cooley, T. P., Kunches, L. M., Saunders, C. A., Ritter, J. K., Perkins, C. J., McLaren, C., McCaffrey, R. P., and Liebman, H. A.** "Once-daily administration of 2',3'-dideoxyinosine (ddI) in patients with the acquired immunodeficency syndrome or AIDS-related complex" N. Engl. J. Med. 322:1340-1345 (1990).

161. **Yarchoan, R., Bouvet, E., Casalino, E., Prevost, M. H., and Vachon, F.** "Fatal case of 2',3'-dideoxyinosine-associated pancreatitis" Lancet 336:1515 (1990).

162. **Fitzgibbon, J. E., Howell, R. M., Haberzettl, C. A., Sperber, S. J., Gocke, D. J., and Dubin, D. T.** "Human immunodeficiency virus type 1 pol gene mutations which cause decreased susceptibility to 2',3'-dideoxycytidine" Antimicrob. Agents Chemother. 36:153-157 (1992).

163. **Polsky, B., Barditch-Crovo, P., Vander Horst, C., Flexner, C., and Raasch, R.** "Anti-HIV-1 activity of FLT: preliminary results from a clinical trial" VIII International Conference on AIDS, Amsterdam, 1992. Abstract PoB3025.

164. **Hitchcock, M. J. M.** "Review: antiviral portrait series, number 1. 2',3'-didehydro-2',3'-dideoxythymidine (D4T), an anti-HIV agent." Antiviral Chemistry and Chemotherapy 2:125-132 (1991).

165. **Russell, J. W., Whiterock, V. J., Marrero, D., and Klunk, L. J.** "Disposition in animals of a new anti-HIV agent: 2',3'-didehydro-3'-deoxythymidine" Drug Metab. Disp. 18:153-157 (1990).

166. **Dubinsky, R. M., Yarchoan, R., Dalakas, M., and Broder, S.** "Reversible axonal neuropathy from the treatment of AIDS and related disorders with 2',3'-dideoxycytidine (ddC)" Muscle Nerve 12:856-860 (1989).

167. **Yarchoan, R., Mitsuya, H., Thomas, R. V., Pluda, J. M., Hartman, N. R., Perno, C. F., Marczyk, K. S., Allain, J. P., Johns, D. G., and Broder, S.** "In vivo activity against HIV and favorable toxicity profile of 2',3'-dideoxyinosine" Science 245:412-415 (1989).

168. **Schinazi, R. F., Chu, C. K., Peck, A., McMillan, A., Mathis, R., Cannon, D., Jeong, L.-S., Beach, J. W., Choi, W.-B., Yeola, S., and Liotta, D. C.** "Activity

of the four optical isomers of 2',3'-dideoxy-3'-thiacytidine (BCH-189) against HIV-1 in human lymphocytes" Antimicrob. Agents Chemother. 36:672-676 (1992).

169. **Van Leeuven, R., Boucher, C., Katlama, C., Ingrand, D., and Kitchen, V.** "A phase I/II study of 3TC in HIV positive, asymptomatic or mild ARC patients" Abstract WeB1014. VIII International Conference on AIDS. Amsterdam, 1992.

170. **Schinazi, R. F., Liotta, D. C., Choi, W.-B., Peck, A., McClure, H., Boudinot, F. D., Sommadossi, J. P., Davis, M., Furman, P. A., and Painter, G.** "Selective inhibition of human immunodeficiency virus and hepatitis B virus by 2',3'-dideoxy-5-fluoro-3'-thiacytidine (FTC). National collaborative drug discovery group, frontiers in HIV therapy" San Diego, CA, Nov. 3-7, 1991.

171. **Doong, S. L., Tsai, C. H., Schinazi, R. F., Liotta, D. C., and Cheng, Y. C.** "Inhibition of the replication of hepatitis B virus in vitro by 2',3'-dideoxy-3'-thiacytidine and related analogues" Proc. Natl. Acad. Sci. U. S. A., 88:8495-8499 (1991).

172. **Weiss, R.** "Receptor molecule blocks HIV" Nature (London) 331:15 (1988).

173. **Zimmerman, T. P., Mahony, W. B., and Prus, K. L.** "3'-Azido-3'-deoxythymidine" J. Biol.Chem. 262:5748-5754 (1987).

174. **Griffiths, D. A., Hall, S. D., and Sokol, P. P.** "Interaction of 3'-azido-3'-deoxythymidine with organic ion transport in rat renal basolateral membrane vesicles" J. Pharm. Exp. Ther. 257:149-155 (1991).

175. **Huang, Q. Q., Yao, S. Y. M., Ritzel, M. W. L., Paterson, A. R. P., Cass, C. E., and Young, J. D.** "Cloning and functional expression of a complementary DNA encoding a mammalian nucleoside transport protein" J. Biol. Chem. 269:17757-17760 (1994).

176. **Young, J. D., Huang, Q. Q., Yao, S. Y. M., Ritzel, M. W.** L., **Paterson, A. R. P., Cass, C. E.** "Cloning and functional expression of a cDNA encoding a mammalian sodium-dependent nucleoside transporter selective for adenosine pyrimidine nucleosides and anti-viral pyrimidine nucleoside analogs" Drug Develop. Res. 31:335 (1994).

177. **Domin, B. A., Mahony, W. B., and Zimmerman, T. P.** "2',3'-Dideoxythymidine permeation of the human erythrocyte membrane by nonfacilitated diffusion" Biochem. Biophys. Res. Commun. 154:825-831 (1988).

178. **August, M. E., Birks, E. M., and Prusoff, W. H.** "3'-Deoxythymidin-2'-ene permeation of human lymphocyte H9 cells by nonfaciliated diffusion" Mol. Pharmacol. 39:246-249 (1991).

179. **Plagemann, P. G. W., and Woffendin, C.** "Permeation and salvage of dideoxyadenosine in mammalian cells" Mol. Pharmacol. 36:185-192 (1989).

180. **Plagemann, P. G. W., and Woffendin, C.** "Dideoxycytidine permeation and salvage by mouse leukemia cells and human erythrocytes" Mol. Pharmacol. 38:3469-3475 (1989).

181. **Singer, S. J., and Nicolson, G. L.** "The fluid mosaic model of the structure of cell membrane" Science 175:720-731 (1972).

182. **Cass, C. E.** "Nucleoside transport" in Drug transport in antimicrobial and anticancer chemotherapy N. H. Georgopapadakou (ed.), Marcel Dekker. in press.

183. **Plagemann, P. G. W., and Wohlhueter, R. M.** "Nucleoside transport in cultured mammalian cells. Multiple forms with different sensitivity to inhibition by nitrobenzylthioinosine or hypoxanthine" Biochim. Biophys. Acta 773:39-52 (1984).

184. **Belt, J. A.** "Heterogeneity of nucleoside transport in mammalian cells. Two types of transport activity in L1210 and other cultured neoplastic cells" Mol. Pharmacol. 24:479-484 (1983).

185. **Cass, C. E., Gaudette, L. A., and Paterson, A. R. P.** "Mediated transport of nucleosides in human erythrocytes. specific binding of the inhibitor nitrobenzylthionosine to nucleoside transport sites in the erythrocyte membrane" Biochim. Biophys. Acta 345:1-10 (1974).

186. **Paterson, A. R. P., Kolassa, N., and Cass, C. E.** "Transport of nucleoside drugs in animal cells" Pharmacol. Ther. 12:515 (1981).

187. **Plagemann, P. G. W., and Wohlhueter, R. M.** "Nitrobenzylthioinosine-sensitive and -resistant nucleoside transport in normal and transformed rat cells" Biochim. Biophys. Acta 816:387-395 (1985).

188. **Oliver, J. M., and Paterson, A. R. P.** "Nucleoside transport I. A mediated process in human erythrocytes" Can. J. Biochem. 49:262-270 (1971).

189. **Fleit, H., Conklyn, M., Stebbins, R. D., and Silber, R.** "Function of 5-nucleotidase in the uptake of adenosine from AMP by human lymphocytes" J. Biol. Chem. 250:8889-8892 (1975).

190. **Wiley, J. S., Taupin, J., Jamieson, G. P., Snook, M., Sawyer, W. H., and Finch, L. R.** "Cytosine arabinoside transport and metabolism in acute leukemias and T cell lymphoblastic lymphoma" J. Clin. Invest. 75:632-642 (1985).

191. **Kaplinsky, C., Yeger, H., Estrov, Z., Barankiewicz, J., Pawlin, G., Freedman, M. H., and Cohen, A.** "Selective protection of tubercidin toxicity by nitrobenzyl thioinosine in normal tissues but not in human neuroblastoma cells" Cancer Chemother. Pharmacol. 17: 264-268 (1986).

192. **Crawford, C. R., Ng, C. Y. C., Noel, L. D., and Belt, J. A.** "Nucleoside transport in L1210 murine leukemia cells" J. Biol. Chem. 265:9732-9736 (1990).

193. **Vijayalakshimi, D., and Belt, J. A.** "Sodium-dependent nucleoside transport in mouse intestinal epithelial cells" J. Biol. Chem. 263:19419-19423 (1988).

194. **Jarvis, S. M.** "Characterization of sodium-dependent nucleoside transport in rabbit intestinal brush-border membrane vesicles" Biochim. Biophys. Acta 979:132-138 (1989).

195. **Roden, M., Paterson, A. R. P., and Turnheim, K.** "Sodium-dependent nucleoside transport in rabbit intestinal epithelium" Gastroenterology 100:1553-1562 (1991).

196. **Lee, C. W., Cheeseman, C. I., and Jarvis, S. M.** "Transport characteristics of renal brush border Na^+- and K^+-dependent uridine carriers" Am. J. Physiol. 258:F1203-1210 (1990).

197. **William, T. C., and Jarvis, S. M.** "Multiple sodium-dependent nucleoside transport systems in bovine renal brush border membrane vesicles" Biochem. J. 274:27-33 (1991).

198. **Gutierrez, M. M., and Giacomini, K. M.** "Substrate selectivity, potential sensitivity and stoichiometry of Na+-nucleoside transport in brush border membrane vesicles from human kidney" Biochim. Biophys. Acta 1149:202-208 (1993).

199. **Wu, X., Yuan, G., Brett, C. M., Hui, A. C., and Giacomini, K. M.** "Sodium-dependent nucleoside transport in choroid plexus from rabbit" J. Biol. Chem. 267:8813-8818 (1992).

200. **Gati, W. P., and Paterson, A. R. P.** in Red blood cell membranes (Agre, P., and Parker, J. C., eds) pp. 635-661, Marcel Dekker Inc., New York. (1989).

201. **Paterson, A. R. P., Clanachan, A. S., Craik, J. D., Gati, W. P., Jakobs, E. S., Wiley, J. S., and Cass, C. E.** In Role of Adenosine and Adenine Nucleotides in

the Biological System (Imai, S., and Nakazawa, M., eds) pp. 133-149, Elsevier, Tokyo, 1991.

202. **Gati, W. P., Paterson, A. R. P., Tyrrell, D. L. J., Cass, C. E., Moravek, J., and Robins, M. J.** "Nucleobase transporter-mediated permeation of 2',3'-dideoxyguanosine in human erythrocytes and human T-lymphoblastoid CCRF-CEM cells" Biochem. Mol. Biol. 267:22272-22276 (1992).

203. **Domin, B. A., Mahony, W. B., and Zimmerman, T. P.** "Membrane permeation mechanisms of 2',3'-dideoxynucleosides" Biochem. Pharmacol. 46:725-729 (1993).

204. **Gati, W. P., Dagnino, L., and Paterson, A. R. P.** "Enantiomeric selectivity of adenosine transport systems in mouse erythrocytes and L1210 cells" Biochem. J. 263: 957-960 (1989).

205. **Kawaguchi, T., Ishikawa, K., Seki, T., and Juni, K.** "Ester prodrugs of zidovudine" J. Pharm. Sci. 79:531-533 (1990).

206. **Aggarwal, S. K., Gogu, S. R., Rangan, S. R. S., and Agrawal, K. C.** "Synthesis and biological evaluation of prodrugs of zidovudine" J. Med. Chem. 33:1505-1510 (1990).

207. **Piantadosi, C., Marasco, C. J. Jr., Morris-Natschke, S. L., Meyer, K. L., Gumus, F., Surles, J. R., Ishaq, K. S., Kucera, L. S., Iyer, N., Wallen, A., Piantadosi, S., and Modest, E. J.** "Synthesis and evaluation of novel ether lipid nucleoside conjugates for anti-HIV-1 activity" J. Med. Chem. 34:1408-1414 (1991).

208. **Hostetler, K. Y., Stuhmiller, L. M., Lenting, H. B. M., and Bosch, H. V. D.** "Synthesis and antiretroviral activity of phospholipid analogs of azidothymidine and other antiviral nucleosides" J. Biol. Chem. 265:6112-6117 (1990).

209. **Stein, J. M., Neto, C. C., Sarin, P. S., Sun, D. K., Sehgal, R. K., and Turcotte, J. G.** "Lipid conjugates of antiretroviral agents. I: Azidothymidine-monophosphate-diglyceride: anti-HIV activity, physical properties, and interaction with plasma proteins" Biochem. Biophys. Res. Comm. 171:451-457 (1990).

210. **Galinsky, R. E., Hoesterey, B. L., and Anderson, B. D.** "Brain and cerebrospinal fluid uptake of zidovudine (AZT) in rats after intravenous injection" Life Sci. 47:781-788 (1990).

211. **Hedaya, M. A., and Sawchuk, R. J.** "Effect of probenecid on the renal and nonrenal clearances of zidovudine and its distribution into cerebrospinal fluid in the rabbits" J. Pharm. Sci. 78:716-722 (1989).

212. **Wong, S. L., Van Belle, K., and Sawchuk, R. J.** "Distributional transport kinetics of zidovudine between plasma and brain extracellular fluid/cerebrospinal fluid in the rabbit: Investigation of the inhibitory effect of probenecid utilizing microdialysis" J. Pharmacol. Exp. Ther. 264:899-909 (1993).

213. Bioreversible carriers in drug design. Theory and application. Roche, E. B. ed. The American Pharmaceutical Association. 1987.

214. **Stella, V.** In "Pro-drugs as novel drug delivery system" American Chemical Society: Washington DC, 1975, pp 1-115.

215. **Silverman, R. B.** in The organic chemistry of drug design and drug action. Academic Press, Inc. pp 352-401.

216. **Pitman, I. H.** "Prodrugs of amides, imides and amines" Med. Res. Rev. 1:189-214 (1981).

217. **Bundgaard, H.** "Novel bioreversible derivatives of amides, imides, ureides, amines and other chemical entities not readily derivatizable" in "Optimization of drug delivery" Bundgaard, H., Hansen, A. B., and Kofod, H. (eds.), Munksgaard, Copenhagen, 1982, pp. 178-197.

218. **Bodor, N.** "Novel approaches in prodrug design" Drugs of the Future" 6:165-182 (1981).

219. **Bundgaard, H.** "Design of prodrugs: Bioreversible derivatives for various functional groups and chemical entities" in "Design of prodrugs" Bundgaard, H. (ed.), Elsevier Biomedical Press, Amsterdam, 1986.

220. **Sloan, K. B., and Bodor, N.** "Hydroxymethyl and acyloxymethyl prodrugs of theophylline: enhanced delivery of polar drugs through the skin" Int. J. Pharm. 12:299-313 (1982).

221. **Sloan, K. B., Hashida, M., Alexander, N., Bodor, N., and Higuchi, T.** "Prodrugs of 5-thiopurines: enhanced delivery through the skin" J. Pharm. Sci. 72:372-378 (1983).

222. **Møllgaard, B., Hoelgaard, A., and Bundgaard, H.** "Pro-drugs as drug delivery systems. XXIII. Improved dermal delivery of 5-fluorouracil through human skin via N-acyloxymethyl pro-drug derivatives" Int. J. Pharm. 12:153-162 (1982).

223. **De Miranda, P., Krasny, H. C., Page, D. A., and Elion, G. B.** "The disposition of acyclovir in different species" J. Pharmacol. Exp. Ther. 219:309-315 (1981).

224. **De Miranda, P., and Blum, M. R.** "Pharmacokinetics of acyclovir after intravenous and oral administration" J. Antimicrob. Chemother. 12 (suppl. B):29-37 (1983).

225. **Van Dyke, R. B., Connor, J. D., Wyborny, C., Hintz, M., and Keeney, R. E.** "Pharmacokinetics of orally administered acyclovir in patients with herpes progenitalis" Am. J. Med. 73:172-175 (1982).

226. **Good, S. S., Krasny, H. C., Elison, G. B., and De Miranda, P.** "Disposition in the dog and the rat of 2,6-diamino-9-(2-hydroxyethoxymethyl)purine (A134U), a potential prodrug of acyclovir" J. Pharmacol. Exp. Therap. 227:644-651 (1983).

227. **Krenitsky, T. A., Hall, W. W., De Miranda, P., Beauchamp, L. M., Schaeffer, H. J., and Whiteman, P. D.** "6-Deoxyacyclovir: A xanthine oxidase-activated prodrug of acyclovir" Proc. Natl. Acad. Sci. U. S. A.. 81:3209-3213 (1984).

228. **Spector, T., Jones, T. E., and Beacham, L. M.** "Conversion of 2,6-diamino-9-(2-hydroxyethoxymethyl)purine to acyclovir as catalyzed by adenosine deaminase" Biochem. Pharmacol. 32:2505-2509 (1983).

229. **Selby, P., Blake, S., Mbidde, E. K., Hickmott, E., Powles, R. L., Stolle, K., McElwain, T. J., Whiteman, P. D., and Fiddian, A. P.** "Amino (hydroxyethoxymethyl) purine: a new well-absorbed prodrug of acyclovir" Lancet, ii, 1428-1430 (1984).

230. **Wade, L. A., and Katzman, R.** "Synthetic amino acids and the nature of L-dopa transport at the blood-brain barrier" J. Neurochem. 25:837 (1975).

231. **Duschinsky, R., Gabriel, T., Tautz, W., Nussbaum, A., Hoffer, M., and Grunberg, E.** "Nucleosides. XXXVII. 5,6-substituted 5-fluorodihydropyrimidines and their 2'-deoxyribonucleosides" J. Med. Chem. 10:47-58 (1967).

232. **Cheraghali, M., Kumar, R., Wang, L., Knaus, E. E., and Wiebe, L. I.** "Synthesis, biotransformation, pharmacokinetics, and antiviral properties of 5-ethyl-5-halo-6-methoxy-5,6-dihydro-2'-deoxyuridine diastereomers" Biochem. Pharmacol. 47:1615-1625 (1994).

233. **Cheraghali, A. M., Kumar, R., Morin, K. W., Knaus, E. E., and Wiebe, L. I.** "Evaluation of (5R,6R)-5-bromo-6-ethoxy-5-ethyl-5,6-dihydro-2'-deoxyuridine" Antimicob. Agents Chemother., in press.

234. **Amidon, G. L.** "Drug derivatization as a mean of solubilization: Physicochemical and biochemical strategies" in Techniques of solubilization of drugs, Yalkowsky, S. H. ed., Marcel Dekker, Inc., New York, pp.183-221, 1981.

235. **Sloan, K. B., Koch, S. A. M., and Siver, K. G.** "Mannich base derivatives of theophylline and 5-fluorouracil: syntheses, properties and topical delivery characteristics" Int. J. Pharm. 21:251-264 (1984).

236. **Daehne, W. V., Frederiksen, E., Gundersen, E., Lund, F., Mørch, P., Petersen, H. J., Roholt, K., Tybring, L., and Godtfredsen, W. O.** "Acyloxymethyl esters of ampicillin" J. Med. Chem. 13:607-612 (1970).

237. **Clayton, J. P., Cole, M., Elson, S. W., and Ferres, H.** "BRL. 8988 (talampicillin), a well-absorbed oral form of ampicillin" Antimicrob. Agents Chemother. 5:670-671 (1974).

238. **Shiobara, Y., Tachibana, A., Sasaki, H., Watanabe, T., and Sado, T.** "Phthalidyl D-α-aminobenzylpenicillinate hydrochloride (PC-183), a new orally active ampicillin eater" J. Antibiot. 27:665-673 (1974).

239. **Bodin, N. O., Ekström., B., Forsgren, U., Jalar, L. P., Magni, L., Ramsey, C. H., and Sjöberg, B.** "Bacampicillin: A new orally well-absorbed derivative of ampicillin" Antimicrob. Agents Chemother. 8:518-525 (1975).

240. **Wilk, S., Mizoguchi, H., and Orlowski, M.** "γ-Glutamyl Dopa: A kidney-specific dopamine precursor" J. Pharmacol. Exp. Ther. 206:227-232 (1978).

241. **Orlowski, M., Mizoguchi, H., and Wilk, S.** "N-Acyl-γ-glutamyl derivatives of sulfamethoxazole as models of kidney-selective prodrugs" J. Pharmacol. Exp. Ther. 212:167-172 (1979).

242. **Buur, A., and Bundgaard, H.** "Prodrugs of 5-fluorouracil. I. Hydrolysis kinetics and physicochemical properties of various N-acyl derivatives of 5-fluorouracil" Int. J. Pharm. 21:349-364 (1984).

243. **Flynn, G., L., and Lamb, D. J.** "Factors influencing solvolysis of corticosteroid-21-phosphate esters" J. Pharm. Sci. 59:1433-1438 (1970).

244. **Kwee, K. S. L., and Stolk, L. M. L.** "Formulation of a stable vidarabine phosphate injection" Pharm. Weekbl. Sci. Ed. 6:101-104 (1984).

245. **Hong, W.-H., and Szulczewski., D. H.** "Stability of vidarabine-5'-phosphate in aqueous solusions" J. Parent. Sci. Techn. 38:60-64 (1984)

246. **Varia, S. A., Schuller, S., and Stella, V. J.** "Phenytoin prodrugs IV: Hydrolysis of various 3-(hydroxymethyl)phenytoin esters" J. Pharm. Sci. 73:1074-1080 (1984).

247. **Posternak, T., Sutherland, E. W., and Henion, W. F.** "Derivatives of cyclic 3',5'-adenosine monophosphate" Biochim. Biophys. Acta 65:558 (1962).

248. **Bodor, N., Farag, H. H., and Brewster, M. E.** "Site-specific, sustained release of drugs to the brain" Science 214:1370-1372 (1981).

249. **Bodor, N., and Brewster, M. E.** "Problems of delivery of drugs to the brain" Pharmacol. Ther. 19:337-386 (1983).

250. **Bodor, N.** "Targeting of drugs to the brain" Methods Enzymol. 112:381-396 (1985).

251. **Bodor, N., Nakamura, T., and Brewster, M. E.** "Improved delivery through biological membranes. Synthesis, distribution, and neurochemical effects of a tryptamine chemical delivery system" Drug Des. Deliv. 1:51-64 (1986).

252. **Brewster, M. E., Ertes, K. S., and Bodor, N.** "Improved delivery through biological membranes. 32. Synthesis and biological activity of brain-targeted delivery systems for various estradiol derivatives" J. Med. Chem. 31:244-249 (1988).

253. **Torrence, P. F., Kinjo, J., Lesiak, K., Balzarini, J., and De Clercq, E.** "AIDS dementia: synthesis and properties of a derivative of 3'-azido-3'-deoxythymidine (AZT) that may become 'locked' in the central nervous system" FEBS Letters 234:135-140 (1988).

254. **Kumar, R., Knaus, E. E., and Wiebe, L. I.** "Synthesis of (E)-5-(2-iodovinyl)-3'-O-(1-methyl-1,4-dihydropyridyl-3-carbonyl)-2'-fluoro-2'-deoxyuridine (IVFRU-CDS) for brain targetted delivery of IVFRU, an antiviral nucleoside" Nucleosides & Nucleotides 12:895-904 (1993).

255. **Morin, K. W., Wiebe, L. I., and Knaus, E. E.** "Synthesis of brain-targeted 1-(2-deoxy-2-fluoro-β-D-ribofuranosyl)-(E)-5-(2-iodovinyl)uracil coupled to a dihydropyridine $\rightleftharpoons$ pyridinium salt redox chemical-delivery system" Carbohy. Res. 249:109-116 (1993).

256. **Rand, K. H., Bodor, N., El Koussi, A. E., Raad, I., Miyake, A., Houk, H., and Gildersleeve.** "Potential treatment of herpes simplex virus encephalitis by brain-specific delivery of trifluorothymidine using a dihydropyridine⇔pyridinium salt type redox delivery system" J. Med. Virol. 20:1-8 (1986).

257. **Chu, C. K., Bhadti, V. S., Doshi, K. J., Etse, J. T., Gallo, J. M., Boudinot, F. D., and Schinazi, R. F.** "Brain targeting of anti-HIV nucleosides: synthesis and in vitro and in vivo studies of dihydropyridine derivatives of 3'-azido-2',3'-

dideoxyuridine and 3'-azido-3'-deoxythymidine" J. Med. Chem. 33:2188-2192 (1990).

258. **Palomino, E., Kessel, D., and Horwitz, J. P.** "A dihydropyrimidine carrier system for sustained delivery of 2',3'-dideoxynucleosides to brain" J. Med. Chem. 32:622-625 (1989).

259. **Miller, W. H., and Miller, R. L.** "Phosphorylation of acyclovir (acycloguanosine) monophosphate by GMP kinase" J. Biol. Chem. 255:7204-7207 (1980).

260. **Furman, P. A., McGuirt, P. V., Keller, P. M., Fyfe, J. A., and Elion, G. B.** "Inhibition by acyclovir of cell growth and DNA synthesis of cells biochemically transformed with herpes virus genetic information" Virology 102:420-430 (1980).

261. **Gray, G. D., Nichol, F. R., Mickelson, M. M., Camiener, G. W., Gish, D. T., Kelly, R. C., Wechter, W. J., Moxley, T. E., and Neil, G. L.** "Immunosuppressive, antiviral and antitumor activities of cytarabine derivatives" Biochem. Pharmacol. 21:465-475 (1972).

262. **Warner, D. T., Neil, G. L., Taylor, A. J., and Wechter, W. J.** "Nucleic acids. 13. 3'-O- and 2'-O-esters of 1-β-D-arabinofuranosylcytosine as antileukemic and immunosuppressive agents" J. Med. Chem. 15:790-792 (1972).

263. **Neil, G. L., Buskirk, H. H., Moxley, T. E., Manak, R. C., Kuentzel, S. L. and Bhuyan, B. K.** "Biochemical and pharmacologic studies with 1-β-D-arabinofuranosylcytosine 5'-adamantoate(NSC-117614), a depot form of cytarabine" Biochem. Pharmacol. 20:3295-3308 (1972).

264. **Gish, D. T., Kelly, R. C., Camiener, G. W., and Wechter, W. J.** "Nucleic acids. 11. Synthesis of 5'-esters of 1-β-D-arabinosylfuranosylcytosine possessing antileukemic and immunosuppressive activity" J. Med. Chem. 14:1159-1162 (1971).

265. **Neil, G. L., Wiley, P. F., Manak, R. C., and Moxley, T. E.** "Antitumor effect of 1-β-D-arabinofuranosylcytosine 5'-adamantoate (NSC 117614) in L1210 leukemic mice" Cancer Res. 30:1047-1054 (1970)

266. **McGuigan, C., Nicholls, S. R., O'Connor, T. J., and Kinchington, D.** "Synthesis of some novel dialkyl phosphate derivatives of 3'-modified nucleosides as potential anti-AIDS drugs" Antiviral Chemistry and Chemotherapy 1:25-33 (1990).

267. **Vazeux, R., Brousse, N., Jarry, A., Henin, D., Marche, C., Vedrenne, C., Mikol, J., Wolff, M., Michon, C., Rozenbaum, W., Bureau, J. F.,**

Montagnier, L., and Brachie, M. "AIDS subacute encephalitis, Identification of HIV-infected cells" Am. J. Pathol. 126:403-410 (1987).

268. **Stoler, M. H., Eskin, T. A., Benn, S., Angerer, R. C., and Angerer, L. M.** "Human T-cell lymphotropic virus type III infection of the central nervous system" J. Am. Med. Assoc. 256:2360-2364 (1986).

269. **Budka, H.** "Multinucleated giant cells in brain:A hall mark of the acquired immune deficiency syndrome (AIDS)" Acta Neuropathol. 69:253-258 (1986).

270. **Black, P.** "HTLV-III, AIDS and the brain" N. Engl. J. Med. 313:1538-1539 (1985).

271. **Vistica, D. T.** "Cytotoxicity as an indicator for transport mechanism: evidence that murine bone marrow progenitor cells lack a high-affinity leucine carrier that transports melphalan in murine" Blood 56:427 (1980).

272. **Balzarini, J., Cooney, D. A., Dalal, M., Kang, G.-J., Cupp, J. E., De Clercq, E., Broder, S., and Johns, D. G.** "2',3'-dideoxycytidine: regulation of its metabolism and anti-retroviral potency by natural pyrimidine nucleosides and by inhibitors of pyrimidine nucleotide synthesis" Mol. Pharmacol. 32:798-806(1987).

273. **Starnes, M. C., and Cheng, Y.-C.** "Cellular metabolism of 2',3'-dideoxycytidine, a compound active against human immunodeficiency virus in vitro" J. Biol. Chem. 262:988-991 (1987).

274. **Hao, Z., Cooney, D. A., Farquhar, D., Perno, C. F., Zhang, K., Masood, R., Wilson, Y., Hartman, N. R., Balzarini, J., and Johns, D. G.** "Potent DNA chain termination activity and selective inhibition of human immunodeficiency virus reverse transcriptase by 2',3'-dideoxyuridine-5'-triphosphate" Mol. Pharmacol. 37:157-163 (1990).

275. **Johnson, M. A., Ahluwalia, G., Connelly, M. C., Cooney, D. A., Broder, S., Johns, D. G., and Fridland, A.** "Metabolic pathways for the activation of the antiretroviral agent 2',3'-dideoxyadenosine in human lymphoid cells" J. Biol. Chem. 263:15354-15357 (1988).

276. **Johnson, M. A., and Fridland, A.** "Phosphorylation of 2',3'-dideoxyinosine by cytosolic 5'-nucleotidase of human lymphoid cells" Mol. Pharmacol. 36:291-295 (1989).

277. **Perno, C. F., Yarchoan, R., Cooney, D. A., Hartman, N. R., Webb, D. S. A., Hao, Z., Mitsuya, H., Johns, D. G., and Broder, S.** "Replication of human immunodeficiency virus in monocytes" J. Exp. Med. 169:933-951 (1989).

278. **Namane, A., Gouyette, C., Fillion, M-P., Fillion, G., and Huynh-Dinh, T.** "Improved brain delivery of AZT using a glycosyl phosphotriester prodrug" J. Med. Chem. 35:3039-3044 (1992).

279. **Henin, Y., Gouyette, C., Schwartz, O., Debouzy, J.-C., Neumann, J.-M., and Huynh- Dinh, T.** "Lipophilic glycosyl phosphotriester derivatives of AZT: Synthesis, NMR transmembrane transport study, and antiviral activity" J. Med. Chem. 34:1830-1837 (1991).

280. **McGuigan, C., Pathirana, R. N., Balzarini, J., and De Clercq, E.** "Intracellular delivery of bioactive AZT nucleotides by aryl phosphate derivatives of AZT" J. Med. Chem. 36:1048-1052 (1993).

281. **Balzarini, J., Herdewijn, P., and De Clercq, E.** "Differential patterns of intracellular metabolism of 2',3'-didehydro-3'-dideoxythymidine (d4T) and 3'-azido-3'-deoxythymidine (AZT), two potent anti-HIV compounds" J. Biol. Chem. 264:6127-6133 (1989).

282. **Mansuri, M. M., Hitchcock, M. J. M., Buroker, R. A., Bregman, C. L., Ghazzouli, I., Desiderio, J. V., Starrett, J. E., Jr., Sterzycki, R. Z., and Martin, J. C.** "Comparison of in vitro biological properties and mouse toxicities of three thymidine analog active against HIV" Antimicrob. Agents Chemother. 34:637-641 (1990).

283. **Ho, H.-T., and Hitchcock, M. J. M.** "Cellular pharmacology of 3'-dideoxy-2',3'-didehydrothymidine, a nucleoside analog active against HIV" Antimicrob. Agents Chemother. 33:844-849 (1989).

284. **Sergheraert, C., Pierlot, C., Tartar, A., Henin, Y., and Lemaitre, M.** "Synthesis and anti-HIV evaluation of d4T and d4T monophosphate prodrugs" J. Med. Chem. 36:826-830 (1993).

285. **Hostetler, K. Y., Richman, D. D., Carson, D. A., Stuhmiller, L. M., van Wijk, G. M. T., and van den Bosch, H.** "Greatly enhanced inhibition of human immunodeficiency virus type 1 replication in CEM and HT 4-6C cells by 3'-deoxythymidine diphosphate dimyristoylglycerol, a lipid prodrug of 3'-deoxythymidine" Antimicrob. Agents Chemother. 36:2025-2029 (1992).

286. **Yarchoan, R., Thomas, R. V., Allain, J. P., McAtee, N., Dubinsky, R., Mitsuya, H., Lawley, S. T., Safai, B., Myers, C. E., Perno, C. F., Klecker, R. W., Wills, R. J., Fischl, M. A., McNeel, M. C., Pluda, J. M., Leuther, M., Collins, J. M., and Broder, S.** "Phase I studies of 2',3'-dideoxycytidine in severe human immunodeficiency virus infection as a single agent and alternating with zidovudine (AZT)" Lancet i: 76-81 (1988).

287. **Bodor, N., Shek, E., and Higuchi, T.** "Delivery of a quaternary pyridinium salt across the blood brain barrier by its dihydropyridinederivative" Science 190:155 (1975).

288. **Vago, L., Castagna, A., Lazzarin, A., Trabattoni, G., Clinque, P., and Costanzi, G.** "Reduced frequency of HIV-induced brain lesions in AIDS patients treated with zidovudine" J. Acquir. Immune. Defic. Syndr. 6:42-45 (1983).

289. **Palomino, E., Kessel, D., and Horwitz, J. P.** "A dihydropyridine carrier system for delivery of 2',3'-dideoxycytidine (DDC) to the brain" Nucleosides Nucleotides 11:1639-1649 (1992).

290. **Brewster, M., Anderson, B. D., and Border, N.** "Brain, blood, and cerebrospinal fluid distribution of an azidothymidine chemical delivery system in rabbits" J. Pharm. Sci. 80:843-846 (1991).

291. **Gogu, S. R., Aggarwal, S. K., Rangan, S. R. S., and Agrawal, K. C.** "A prodrug of zidovudine with enhanced efficacy against human immunodeficiency virus" Biochem. Biophys. Res. Commun. 160:656-661 (1989).

292. **Little, R., Bailey, D., Brewster, M. E., Estes, K. S., Clammons, R. M., Saab, A., and Bodor, N.** "Improved delivery through biological membranes. XXXIII. Brain-enhanced delivery of azidothymidine (AZT)" J. Biopharm. Sci. 1:1-18 (1990).

293. **Torrence, P. F., Kinjo, J., Khamnei, S., and Greig, N. H.** "Synthesis and pharmacokinetics of a dihydropyridine chemical delivery system for the antiimmunodeficiency virus agent dideoxycytidine" J. Med. Chem. 36:529-537 (1993).

294. **Lupia, R. H., Ferencz, N., Lertora, J. J. L., Aggarwal, S. K., George, W. J., and Agrawal, K. C.** "Comparative pharmacokinetics of two prodrugs of zidovudine in rabbits: Enhanced levels of zidovudine in brain tissue" Antimicrob. Agents Chemother. 37:818-824 (1993).

295. **Klecker, R. W., Collins, J. M., Yarchoan, R. C., Thomas, R., McAtee, N., Broder, S., and Myers, C. E.** "Pharmacokinetics of 2',3'-dideoxycytidine in patients with AIDS and related disorders" J. Clin. Pharmacol. 28:837-842 (1988).

296. **Kelley, J. A., Litterst, C. L., Roth, J. S., Vistica, D. T., Poplack, D. G., Conney, D. A., Nadkarni, M., Balis, F. M., Broder, S., and Johns, D. G.** "The disposition and metabolism of 2',3'-dideoxycytidine, an in vitro inhibitor of human T-lymphotropic virus type III infectivity, in mice and monkeys" Drug Metab. Dispos. 15:595-601 (1987).

297. **Tomaszewski, J. E., Grieshaber, C. K., Balzarini, J., Johns, D. G., Smith, A. C., Liao, J. T., and Collins, W. T., Jr.** "Toxicologic and pharmacokinetic evaluation of 2',3'-dideoxycytidine (ddCyd., NSC-606170), a potential drug to treat AIDS" Proc. Am. Assoc. Cancer Res. 28:440 (1987).

298. **Collins, J. M., Klecker, R. W., Kelley, J. A., Roth, J. S., McCully, C. L., Balis, F., and Poplack, D. G.** "Pyrimidine dideoxyribonucleosides: selectivity of penetration into cerebrospinal fluid" J. Pharmacol. Exp. Ther. 245:466-470 (1988).

299. **Kucera, L. S., Iyer, N., Leake, E., Raben, A., Modest, E., Daniel, L. W., and Piantadosi, C.** "Novel membrane-interactive ether lipid analogs that inhibit infectious HIV-1 production and induce defective virus formation" AIDS Res. Hum. Retroviruses 6:491-501 (1990).

300. **Kerr, S. G., and Kalman, T. I.** "Highly water-soluble lipophilic prodrugs of the anti-HIV nucleoside analogue 2',3'-dideoxycytidine and its 3'-fluoro derivative" J. Med. Chem. 35:1996-2001 (1992).

301. **Shanmuganathan, K., Koudriakova, T., Nampalli, S., Du, J., Gallo, J. M., Schinazi, F., and Chu, C. K.** "Enhanced brain delivery of an anti-HIV nucleoside 2'-F-ara-ddI by xanthine oxidase mediated biotransformation" J. Med. Chem. 37:821-827 (1994).

302. **Lindsay, S., Liu, T. H., Xu, J. A., Marshall, P. A., Thompson, J. K., Parks, D. A., Freeman, B. A., Hsu, C. Y., and Beckman, J. S.** "Role of xanthine dehydrogenase and oxidase in focal cerebro ischemic injury to rat" Am. J. Physiol. 261:H2051-2057 (1991).

303. **Morin, K., Wiebe, L. I. and Knaus, E. E.** Unpublished results.

304. **Fujita, T., Iwasa, J., and Hansch, C.** "A new substituent constant, π, derived from partition coefficients" J. Am. Chem. Soc. 86:5175-5180 (1964).

305. **Gati, W. P., Knaus, E. E., and Wiebe, L. I.** "Interaction of 2'-halogeno-2'-deoxyuridines with the human erythrocyte nucleoside transport mechanism" Mol. Pharmacol. 23:146-152 (1983).

306. **Gati, W. P., Misra, H. K., Knaus, E. E., and Wiebe, L. I.** "Structural modifications at the 2'- and 3'- positions of some pyrimidine nucleosides as determinants of their interaction with the mouse erythrocyte nucleoside transporter" Biochem. Pharmacol. 33:3325-3331 (1984).

307. **Hanes, C. S.** "CXLVII. Studies on plant amylases. I. The effect of starch concentration upon the velocity of hydrolysis by the amylase of germinated barley" Biochem. J. 26:1406-1421 (1932).

308. **Dixon, M.** "The determination of enzyme inhibitor constants" Biochem. J. 55:170-171 (1953).

309. **Gati, W.** personal communication.

310. **Schwartz, M.** "Thymidine phosphorylase from Escherichia coli" Methods Enzymol. 51:442-445 (1978).

311. **Whittaker, V. P., and Barker, L. A.** "The subcellular fractionation of brain tissue with special reference to the preparation of synaptosomes and their component organelles" in Methods of Neurochemistry vol. 2. pp 1-52, New York, Marcel Dekker, Fried, 1972.

312. **Lowry, O. H., Rosenbrough, N. J., and Randall, R. J.** "Protein measurement with Folin phenol reagent" J. Biol. Chem. 193:265-275 (1951).

313. **Kumar, R., Wang, L., Wiebe, L. I., and Knaus, E. E.** "Synthesis and anti-HIV activity of 5-halo-6-alkoxy (or azido)-5,6-dihydro-3'-azido-3'-deoxythymidine diastereomers as potential prodrugs to 3'-azido-3'-deoxythymidine (AZT)" J. Med. Chem., in press.

314. **Kumar, R., Wiebe, L. I., and Knaus, E. E.** "Synthesis and anti-HIV activity of 5-halo-6-alkoxy-5,6-dihydro-2',3'-didehydro-2',3'-dideoxythymidine diastereomers as potential prodrugs to d4T", in preparation.

315. **Kumar, R., Wang, L., Wiebe, L. I., and Knaus, E. E.** "Synthesis and antiviral (HIV-1, HBV) activities of 5-halo-6-methoxy (or azido)-5,6-dihydro-3'-fluoro-3'-deoxythymidine diastereomers. Potential prodrugs to 3'-fluoro-3'-deoxythymidine (FLT)" J. Med. Chem., 37:3554-3560 (1994).

316. **Blanchard, H., James, M. N. G., Kumar, R., Wiebe, L. I., and Knaus, E. E.** "Structure of (+)-(5R,6R)-5-chloro-6-methoxy-5,6-dihydro-1-(2',3'-didehydro-2',3'-dideoxy-B-D-glycero-2'-enopentofuranosyl)thymine" Acta Cryst. C49:246-250 (1993).

317. **Weislow, O. W., Kiser, R., Fine, B., Bader, J., Shoemaker, R. H., and Boyd, M. R.** "New soluble-formazan assay for HIV-1 cytopathic effects: Application to high-flux screening of synthetic and natural products for AIDS-antiviral activity" J. Natl. Cancer Inst. 81:577-586 (1989).

318. **Rork, G. S., and Pitman, I. H.** "A kinetic study of the dehalogenation of 5-halo-5,6-dihydrouracils in aqueous solutions of sodium bisulfite" J. Am. Chem. Soc. 97:5566-5572 (1975).

319. **Sedor, F. A., and Sander, E. G.** "Dehalogenation of 5-bromo-6-methoxy-5,6-dihydrothymine by cysteine" J. Am. Chem. Soc. 98:2314-2319 (1976).

320. **Rork, G. S., and Pitman, I. H.** " A kinetic study of the dehalogenation of 5-chloro, 5-bromo, and 5-iodouracil in aqueous solutions of sodium bisulfite" J. Am. Chem. Soc. 97:5559-5565 (1975).

321. **Sedor, F. A., Jacobson, D. G., and Sander, E. G.** "Dehalogenation of 5-bromouracil by bisulfite buffers. Kinetic evidence for a multistep reaction pathway" J. Am. Chem. Soc. 97:5572-5577 (1975).

322. **Sander, E. G., and Deyrup, C. L.** "The effect of bisulfite on the dehalogenation of 5-chloro-, 5-bromo-, and 5-iodouracil" Arch. Biochem. Biophys. 150:600-605 (1972).

323. **Wataya, Y., Negishi, K., and Hayatsu, H.** "Debromination of 5-bromo-2'-deoxyuridine by cysteine. Formation of deoxyuridine and S-[5-(2'-deoxyuridyl)]cysteine" Biochemistry 12:3992-3998 (1973).

324. **Pardridge, W. M.** "Strategies for drug delivery through the blood-brain barrier" Neurobiol. Aging. 10:636-637 (1989).

325. **Stein, W. D.** "Transport and diffusion across cell membrane" pp. 72-77. Academic Press, Orlando, FL.,1986.

326. **Hansch, C., Leo, A., Unger, S. H., Kim, K. H., Nikaitani, D., and Lien, E. J.** "Aromatic substituent constants for structure-activity correlations" J. Med. Chem. 16:1207-1216 (1973).

327. **Hansch, C., and Fujita, T.** "r-s-p Analysis. A method for the correlation of biological activity and chemical structure" J. Am. Chem. Soc. 86:1616-1626 (1964).

328. **Collander, R.** "The permeability of nitella cells to non-eiectrolytes" Physiol. Plant 7:420-445 (1954).

329. **Milborrow, B. V., and Williams, D. A.** "A re-examination of the penetration of nitella cells by non-electrolytes" Physiol. Plant 21:902-909 (1968).

330. **Hansch, C., Muir, R. M., Fujita, T., Maloney, P. P., Geiger, C. F., and Streich, M. J.** "The correlation of biological activity of plant growth regulators

and chloromycetin derivatives with Hammett constants and partition coefficients" J. Am. Chem. Soc. 85:2817-2824 (1963).

331. **Levin, V. N.** "Relationship of octanol/water partition coefficient and molecular weight to rat brain capillary permeability" J. Med. Chem. 23:682-684 (1980).

332. **Balzarini, J., Cooks, M., and De Clercq, E.** "Estimation of the lipophilicity of anti-HIV nucleoside analogues by determination of the partition coefficient and retention time on a Lichrospher 60 RP-8 HPLC column" Biochem. Biophys. Res. Commun. 158:413-422 (1989).

333. **Kong, X.-B., Zhu, Q.-Y., Vidal, P. M., Watanabe, K. A., Polsky, B., Armstrong, D., Ostrander, M., Lang, S. A. Jr., Muchmore, E., and Chou, T.-C.** "Comparisons of anti-human immunodeficiency virus activities, cellular transport, and plasma and intracellular pharmacokinetics of 3'-fluoro-3'-deoxythymidine and 3'-azido-3'-deoxythymidine" Antimicrob. Agents Chemother. 36:808-818 (1992).

334. **Szebeni, J., Wahl, S. M., Popovic, M., Wahl, L. M., Gartner, S., Fine, R. L., Skaleric, U., Friedmann, R. M., and Weinstein, J. N.** "Dipyridamole potentiates the inhibition by 3'-azido-3'-deoxythymidine and other dideoxynucleosides of human immunodeficiency virus replication in monocyte-macrophages" Proc. Natl. Acad. Sci. U. S. A. 86:3842-3846 (1989).

335. **Weinstein, J., Bunow, B., Weislow, O., Schinazi, R. F., Wahl, S. M., Wahl, L. M., and Szebeni, J.** "Synergistic drug combinations in AIDS therapy: dipyridamole/3'-azido-3'-deoxythymidine in particular and principles of analysis in general" Ann. NY. Acad. Sci. 616:367-384 (1990).

336. **Cass, C. E., and Paterson, A. R. P.** "Mediated transport of nucleosides in human erythrocytes. Accelerative exchange diffusion of uridine and thymidine and specificity toward pyrimidine nucleosides as permeants" J. Biol. Chem. 247:3314-3320 (1972).

337. **Cass, C. E., and Paterson, A. R. P.** "Mediated transport of nucleosides by human erythrocytes, specificity toward purine nucleosides as permeants" Biochim. Biophys. Acta 291:734 (1973).

338. **Cabantchik, Z. I., and Ginsburg, H.** "Transport of uridine in human red blood cells. Demonstration of a simple carrier-mediated process" J. Gen. Physiol. 69:75-96 (1975).

339. **Plagemann, P. G. W., Wohlhueter, R. M., and Erbe, J.** "Nucleoside transport in human erythrocytes" J. Biol. Chem. 257:12069-12074(1982).

340. **Jarvis, S. M., Hammond, J. R., Paterson, A. R. P., and Clanachan, A. S.** "Nucleoside transport in human erthrocytes" Biochem. J. 210:457-461(1983).

341. **Xu, L.-H.** "Membrane transport and partition of novel pyriimidine nucleosides" M. Sc. Thesis, University of Alberta (1988).

342. **Betageri, G. V., Szebeni, J., Hung, K., Patel, S. S., Wahl, L. M., Corcoran, M., Weinstein, J. N.** "Effect of dipyridamole on transport and phosphorylation of thymidine and 3'-azido-3'-deoxythymidine in human monocyte/macrophages" Biochem. Pharmacol. 40:867-870 (1990).

343. **Cornish-Bowden, A.** "A simple graphical method for determining the inhibition constants of mixed, uncompetitive and non-competitive inhibitors" Biochem. J. 137:143-144 (1974).

344. **Birnie, G. D., Kroeger, H., and Heidelberger, C.** "Studies of fluorinated pyrimidines. VIII. The degradation of 5-fluoro-2'-deoxyuridine and related compounds by nucleoside phosphorylase" Biochem. 2:566-572 (1963).

345. **Samuel, J., Gill, M. J., Iwashina, T., Tovell, D. R., Tyrell, D. L., Knaus, E. E., and Wiebe, L. I.** "Pharmacokinetics and metabolism of E-5-(2-[^{131}I]iodovinyl)-2'-deoxyuridine in dogs" Antimicrob. Agents Chemother. 29:320-324 (1986).

346. **Woodman, P. W., Sarrif, A. M., and Heidelberger, C.** "Inhibition of nucleoside phosphorylase cleavage of 5-fluoro-2'-deoxyuridine by 2,4-pyrimidindione derivatives" Biochem. Pharmacol. 29:1059-1063 (1980).

347. **Veres, Z., Neszmélyi, A., Szabolcs, A., and Dénes, G.** "Inhibition of uridine phosphorylase by pyrimidine nucleoside analogues and consideration of substrate binding to the enzyme based on solution conformation as seen by NMR spectroscopy" Eur. J. Biochem. 178:173-181 (1988).

348. **Niedzwicki, J. G., E. L. Kouni, M.H., Chu, S.H., and Cha, S.** "Structure - activity relationship of ligands of the pyrimidine nucleoside phosphorylase" Biochem. Pharmacol. 32:399-415 (1983).

349. **Veres, Z., Szabolcs, A., Szinai, I, Denes, G., and Jeney, A.** "Enzymatic cleavage of 5-substituted-2'-deoxyuridines by pyrimidine nucleoside phosphorylase" Biochem. Pharmacol. 35:1057-1059 (1986).

350. **Krenitsky, T. A., Barclay, M., and Jacquez, J. A.** "Specificity of mouse uridine phosphorylase" J. Biol. Chem. 239:805-812 (1964).

351. **Niedzwicki, J. G., el Kouni, M. H., Chu, S. H. and Cha, S.** "Pyrimidine acyclonucleosides, inhibitors of uridine phosphorylase" Biochem. Pharmacol. 30:2097-2101 (1981).

352. **Gaitonde, M. K.** "A spectrophotometric method for the direct determination of cysteine in the presence of other naturally occuring amino acids" Biochem. J. 104:627-633 (1967).

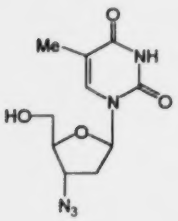
353. **Buhl, R., Jaffe, H. A., Holroyd, K. J., Wells, F. B., Mastrangeli, A., Saltini, C., Cantin, A. M., and Crystal, R. G.** "Systemic glutathione deficiency in symptom-free HIV-seropositive individuals" Lancet 2:1294-1298 (1989).

354. **Fischl, M. A., Parker, C. B., Pettinelli, C., Wulfsohn, M., Hirsch, M. S., et al.** "A randomised controlled trial of reduced daily dose zidovudine in patients with the acquired immunodeficiency syndrome. N. Engl. J. Med. 323:1009-1014 (1990).

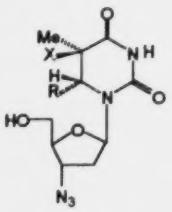
355. **Dischino, D. D., Welch, M. J., Kilbourn, M. R., and Raichle, M. E.** "Relationship between lipophilicity and brain extraction of C-11 labeled radiopharmaceuticals" J. Nucl. Med. 24:1030-1038 (1983).

Appendix

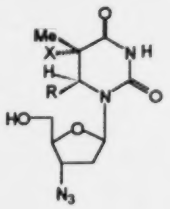
Table 1. *In vitro* anti-HIV activity and selectivity of 5-halo-6-alkoxy (or azido)-5,6-dihydro analogs of AZT in HIV-1 infected CEM cells.



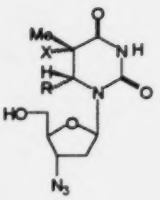
AZT



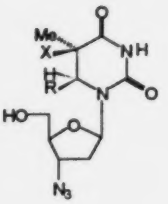
1,3,5,7,9,12-18,20
trans-(+)-(5R,6R)



2,6,8,10,19,21
trans-(-)-(5S,6S)



4,11,22
cis-(+)-(5S,6R)



23
cis-(-)-(5R,6S)

No.	X	R	Configuration	IC_{50} (M)[a]	EC_{50} (M)[b]	T. I.[d]
1	Br	OCH_3	5R,6R	1.72×10^{-5}	3.27×10^{-9}	5260
2	Br	OCH_3	5S,6S	4.25×10^{-5}	2.80×10^{-7}	152
3,4[c]	Cl	OCH_3	5R,6R; 5S,6R	$>8.98 \times 10^{-4}$	5.79×10^{-6}	155
5	I	OCH_3	5R,6R	1.87×10^{-5}	3.17×10^{-9}	5899
6	I	OCH_3	5S,6S	6.42×10^{-6}	5.15×10^{-9}	1246
7	Br	OC_2H_5	5R,6R	1.85×10^{-5}	6.75×10^{-9}	2741
8	Br	OC_2H_5	5S,6S	2.22×10^{-5}	2.37×10^{-8}	937
9	Cl	OC_2H_5	5R,6R	$> 2.00 \times 10^{-4}$	Inactive	ND
10	Cl	OC_2H_5	5S,6S	$> 2.00 \times 10^{-4}$	Inactive	ND
11	Cl	OC_2H_5	5S,6R	$> 2.00 \times 10^{-4}$	Inactive	ND
12	Br	$OCH(CH_3)_2$	5R,6R	$> 2.00 \times 10^{-4}$	5.72×10^{-6}	35
13	Cl	$OCH(CH_3)_2$	5R,6R	$> 2.00 \times 10^{-4}$	Inactive	ND
14	Br	$O(CH_2)_7CH_3$	5R,6R	1.24×10^{-5}	8.56×10^{-7}	14
15	Cl	$O(CH_2)_7CH_3$	5R,6R	3.75×10^{-5}	Inactive	ND
16	Br	$O(CH_2)_{15}CH_3$	5R,6R	1.10×10^{-5}	Inactive	ND
17	Cl	$O(CH_2)_{15}CH_3$	5R,6R	3.10×10^{-5}	Inactive	ND
18,19[c]	Br	N_3	5R,6R;5S,6S	1.76×10^{-5}	2.0×10^{-4}	< 1
20-23[c]	Cl	N_3	5R,6R; 5S,6S; 5S,6R;5R,6S	3.5×10^{-4}	1.49×10^{-6}	235
AZT				5×10^{-4}	3×10^{-9}	>> 10,000

continued

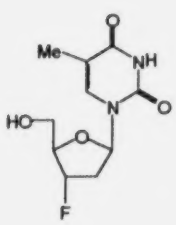
[a] The IC_{50} value is the test drug concentration which results in a 50% survival of uninfected untreated control CEM cells (e.g. cytotoxicity of the test drug).

[b] The EC_{50} value is the test drug concentration which produces a 50% survival of HIV-1 infected cells relative to uninfected untreated controls (e.g. in vitro anti-HIV activity).

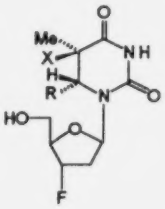
[c] Tested as a mixture of diastereomers.

[d] Therapeutic index = IC_{50}/EC_{50}.

Table 2. *In vitro* anti-HIV activity and selectivity of 5-halo-6-methoxy (or azido)-5,6-dihydro analogs of FLT in HIV-1 infected CEM cells.

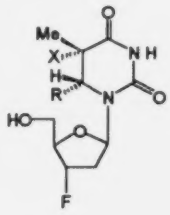


FLT



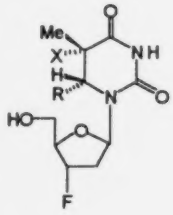
1,3,5,7

trans-(+)-(5R,6R)



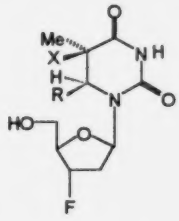
2,6,8

trans-(-)-(5S,6S)



4

cis-(+)-(5S,6R)



9

cis-(-)-(5R,6S)

No.	X	R	Configuration	IC_{50} (M)[a]	EC_{50} (M)[b]	T. I[d]
1	Br	OCH_3	5R,6R	1.72×10^{-6}	5.25×10^{-9}	327
2	Br	OCH_3	5S,6S	9.72×10^{-6}	3.25×10^{-9}	2990
3	Cl	OCH_3	5R,6R	$> 8.0 \times 10^{-4}$	5.55×10^{-6}	144
4	Cl	OCH_3	5S,6R	$> 8.0 \times 10^{-4}$	3.79×10^{-6}	211
5	I	OCH_3	5R,6R	5.73×10^{-5}	5.00×10^{-5}	1.15
6	I	OCH_3	5S,6S	1.22×10^{-5}	3.75×10^{-9}	3253
7,8,9[c]	Br	N_3	5R,6R;5S,6S; 5R,6S	1×10^{-4}	1.45×10^{-8}	7143
FLT					1×10^{-9}	ND

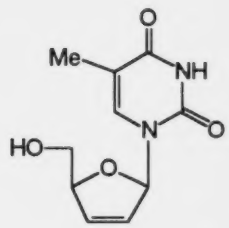
[a] The IC_{50} value is the test drug concentration which results in a 50% survival of uninfected untreated control CEM cells (e.g. cytotoxicity of the test drug).

[b] The EC_{50} value is the test drug concentration which produces a 50% survival of HIV-1 infected cells relative to uninfected untreated controls (e.g. in vitro anti-HIV activity).

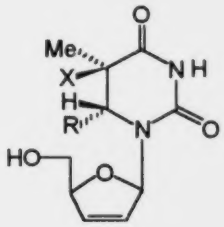
[c] Tested as a mixture of diastereomers.

[d] Therapeutic index = IC_{50}/EC_{50}.

Table 3. In vitro anti-HIV activity and selectivity of 5-halo-6-methoxy-5,6-dihydro analogs of d4T in HIV-1 infected CEM cells.

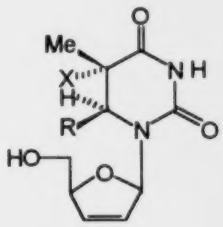


d4T



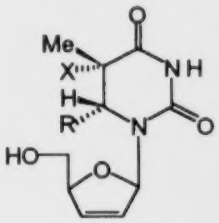
1,3,5

trans-(+)-(5R,6R)



2,4

trans-(-)-(5S,6S)



6

cis-(+)-(5S,6R)

No.	X	R	Configuration	IC_{50} (M)[a]	EC_{50} (M)[b]	T. I.[d]
1,2[c]	Br	OCH_3	5R,6R; 5S,6S	$> 1.28 \times 10^{-4}$	5.46×10^{-5}	2.34
3,4[c]	Cl	OCH_3	5R,6R; 5S,6R	$> 1.03 \times 10^{-3}$	3.75×10^{-4}	2.75
5,6[c]	I	OCH_3	5R,6R; 5S,6S	6.6×10^{-5}	3.76×10^{-7}	176
d4T				3×10^{-4}	1.20×10^{-7}	25

[a] The IC_{50} value is the test drug concentration which results in a 50% survival of uninfected untreated control CEM cells (e.g. cytotoxicity of the test drug).

[b] The EC_{50} value is the test drug concentration which produces a 50% survival of HIV-1 infected cells relative to uninfected untreated controls (e.g. in vitro anti-HIV activity).

[c] Tested as a mixture of diastereomers.

[d] Therapeutic index = IC_{50}/EC_{50}.